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K1-20 KEYNOTE SPEAKER PRESENTATIONS

MOLECULAR AND IMMUNO ADVANCES

K1

The multi-faceted roles of the PI3K-AKT pathway in melanoma

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Background: The PI3K-AKT pathway has been shown to complement activation of the MAPK pathway in melanocyte transformation in preclinical models. We have utilized clinical specimens and cell lines to examine the regulation, functional role, and clinical significance of the PI3K-AKT pathway in melanoma.
Materials and methods: All analyses of clinical specimens were performed under protocols approved by the MD Anderson Institutional Review Board. Identities of cell lines were confirmed by STR-fingerprinting, and cell lines were genotyped by sequenom analysis for hotspot mutations. Reverse phase protein array (RPPA) analysis was performed by the MD Anderson RPPA Core Facility.
Results: Proteomic analysis of frozen melanoma clinical specimens and cell lines demonstrated that phosphorylation (activation) of AKT (P-AKT) correlated inversely with PTEN protein expression. Confirmatory analysis of PTEN expression by an immunohistochemical (IHC) assay on archival tumor samples identified a linear correlation between PTEN measurement by IHC and RPPA. Significantly increased expression of P-AKT was observed in melanomas with complete loss of PTEN protein expression compared to tumors with any other pattern of PTEN expression. Integrated analysis of BRAF/NRAS mutation status, PTEN expression, and clinical outcomes was performed using lymphadenectomy specimens from patients who underwent surgery for stage IIIB/C melanoma. Complete loss of PTEN expression correlated with shorter overall survival and time to brain metastasis on univariate and multivariate analyses. Testing of human melanoma cell lines demonstrated that cells with baseline or compensatory activation of the PI3K-AKT pathway were generally resistant to apoptosis induction following BRAF or MEK inhibition. Extended molecular profiling found that a subset of melanoma cell lines with de novo and acquired resistance to MEK inhibitors are characterized by increased expression of PGC1alpha and a metabolic phenotype of high oxidative phosphorylation (OxPhos). The resistant high OxPhos cell lines all demonstrated synergistic growth inhibition and apoptosis induction with combined targeting of MEK and mTORC1/2. mTORC1/2 inhibition decreased PGC1alpha expression in vitro and in vivo, and caused cytosolic localization of MITF. Treatment with a MEK inhibitor and an mTORC1/2 inhibitor demonstrated marked synergy in a BRAF-mutant, MEK inhibitor resistant human melanoma cell line in vivo.
Conclusions: Activation of the PI3K-AKT pathway correlates with clinical outcomes and resistance to MAPK pathway inhibitors in melanoma. These findings support the rationale to determine the clinical benefit of inhibiting the PI3K-AKT pathway in this disease.

K2

Evolution of resistance to MAPK-targeted therapies

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BRAF inhibitors (BRAFi) elicit rapid antitumor responses in the majority of patients with ^{V600}BRAF mutant melanoma, but acquired resistance is almost universal. Early understanding of how melanomas acquire resistance to BRAFi via MAPK pathway reactivation has guided the development of specific BRAFi-anchored inhibitor combinations designed to overcome resistance. The first of these successful combinations has been between BRAF and MEK inhibitors. Combined BRAF/MEK targeted therapy improves upon BRAFi therapy but is still beset by acquired resistance. Thus, studying how melanomas escape from BRAFi and how these processes are similar or distinct from acquired resistance mechanisms to BRAFi+MEKi remains of the utmost importance to melanoma therapeutics. Recent whole-exome analysis of patient-paired melanoma samples obtained pre-BRAFi treatment and post-disease progression after initial responses have provided landscape genetic perspectives into the nature of tumor heterogeneity, clonal evolution, and core resistance pathways. This benchmark understanding is helping to guide and prioritize clinical studies of BRAFi-based combinations such as that between BRAFi and AKTi. Recent work on the genetic mechanisms of acquired BRAFi+MEKi resistance has shed key insights into the molecular limitations of this therapeutic approach but also novel therapeutic opportunities. Overall, genetic alterations affecting individual genes are not highly recurrent and collectively cannot account for a significant subset of clinical acquired resistance to BRAF or combined BRAF/MEK targeted therapies (MAPKi). Thus, understanding the entire spectrum of genetic and non-genetic mechanisms of acquired MAPKi resistance and the temporal continuum of these evolutionary processes promises to usher in a new era of personalized medicine for melanoma patients.

K3

ErbB3 plays a key role in the early phase of establishment of resistance to BRAF and/or MEK inhibitors

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Background: A major issue in the management of cancer is the development of drug resistance. In metastatic melanoma bearing V600 mutations in the BRAF oncogene, all patients undergo disease relapse after combination therapy with BRAF and MEK inhibitors. Hence, understanding the mechanisms at the basis of development of resistance is fundamental to the discovery of new therapeutic approaches. In our group we have spent the last years to identify mechanisms of early adaptation of BRAF mutated melanoma to BRAF and or MEK inhibitors. We have recently shown that the ErbB3 receptor is involved in the activation of an early feedback survival loop upon cell exposure to BRAF and/or MEK inhibitors. Upregulation of pErbB3, due to enhanced production of its ligand neuregulin-1 (HRG), causes increased AKT phosphorylation and cell survival. Furthermore, we demonstrated that activation of the ErbB3/AKT axis is abrogated by co-treatment with anti-ErbB3 mAbs previously generated in our laboratory.

Materials and methods: Eleven different melanoma cell lines bearing BRAF V600E or BRAF V600D or BRAF V600R mutations were exposed to short term or long term treatment with vemurafenib and/or trametinib and/or anti ErbB3 antibodies A3 and A4. Short term growth inhibition was measured by colony forming assays, cell cycle and apoptosis markers. Long term treatments allowed the selection of resistant clones. Western blot analysis was performed on total protein extracts using anti-ErbB3, anti-AKT and anti-ERK 1/2 antibodies. Mouse xenograft studies were carried out with M14 cells injected s.c. at the dose of 5×10^6 cells. Individual or combined drug treatments began when tumors reached a mean volume of 100 mm^3 and tumor growth was measured by caliper.

Results: We show that ErbB3 undergoes a strong upregulation of its phosphorylation in the absence of external addition of neuregulin (HRG) upon exposure to vemurafenib or trametinib or both drugs in the 10 out of 11 of cell lines tested. Phospho ErbB3 activation is accompanied by strong phosphorylation of downstream AKT. Most importantly anti-ErbB3 monoclonal antibodies combination strongly enhances the ability of BRAF/MEK inhibitors to silence the oncogenic MAPK and AKT pathways. This results in potentiation of growth inhibition and of apoptosis compared to single antibody treatments. Moreover ErbB3 mAbs impair the establishment of resistance and restore drug sensitivity to vemurafenib in resistant melanoma cells. Finally anti-ErbB3 mAbs A3 and A4 combination strongly affect *"in vivo"* melanoma cell growth and reduces tumor relapse when combined with vemurafenib and tramentinib.

Conclusions: Feedback activation of ErbB3/AKT phosphorylation is a fast and common response of melanoma cells to BRAF and/or MEK inhibitors. Here, we show for the first time that the ErbB3 receptor is a key-player also in long-time drug establishment of resistance. These data strongly underline the role of ErbB3 in the rebound of melanoma cell growth following vemurafenib/trametinib treatments and pave the way for the use of anti-ErbB3 mAbs as adjuncts to current target therapies in order to obtain a durable control of tumor growth.

K4

Molecular enhancement of sentinel node evaluation

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Lymphatic mapping and sentinel node biopsy are widely used to stage and manage patients with intermediate and thicker primary cutaneous melanoma. *Likelihood* of sentinel node metastases can be estimated from patients' demography and primary tumor characteristics, but is precisely *determined* only by microscopic evaluation of excised node(s). Molecular/Genetic techniques, such as RT-PCR, Gene Expression Microarray, detection of metastasis-associated gene signatures and gene sequencing are likely to increase precision in future. Recent 10 year results of Multicenter Selective Lymphadenectomy Trial 1 (MSLT1) [1] confirm that biopsy-based management prolongs disease free survival for all node-positive patients (P=0.01-0.03) and significantly increases 10 year distant disease-free survival (P=0.006) and melanoma-specific survival (P=0.02) for node-positive patients with intermediate thickness primaries. The trial also confirmed very clearly that the presence or absence of sentinel node metastases is best determined by close pathological evaluation of sections from nodal tissues, stained conventionally and by immunohistochemistry (S-100 protein, Mart-1, HMB-45). Patients with sentinel node metastases have a significantly unfavorable outcome that correlates with the amount and disposition of nodal tumor as assessed by microscopy, immunohistochemistry and morphometry [2]. Here again precision will be increased by the parallel application of molecular and genetic tests. The accuracy of appropriate patient assignment to early surgical management will be dramatically increased by the addition of molecular testing, more precisely identifying the patients most likely to respond to standard and evolving therapies.

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K5

Mechanisms of synergy of radiotherapy and immunotherapy

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The success of immune checkpoint inhibitors in inducing tumor regression has demonstrated that specific inhibitory pathways are dominant rate-limiting steps in a significant number of patients with melanoma and other advanced cancers. However, in the majority of patients tumor rejection is hindered by multiple immunosuppressive mechanisms present in the tumor microenvironment. Obstacles to immune-mediated tumor control can be present at both the priming and effector phase of the anti-tumor response, and include defective function and activation of antigen-presenting cells, defective T cell recruitment and infiltration of tumors, and defective recognition and killing of cancer cells by T cells. Ionizing radiation therapy (RT) applied locally to a tumor at therapeutic doses has multiple effects that can potentially overcome each of these obstacles, and we have shown that RT is synergistic with immunotherapy. In pre-clinical models RT converted tumors unresponsive to anti-CTLA-4 mAb into responsive ones, achieving

rejection of the irradiated tumor and non-irradiated metastases (abscopal effect) and improved survival [1,2]. At the effector phase, RT enhanced recruitment of activated T cells to the tumor by induction of chemokines [3], and enhanced immune synapse formation between CD8 T and tumor cells by induction of NKG2D ligands [4].

To test the hypothesis that successful tumor rejection induced by RT +anti-CTLA-4 requires a significant change in the quantity and quality of tumor-infiltrating lymphocytes (TILs) we performed a comprehensive evaluation of the breadth and depth of the T cell repertoire modulated by treatment using high-throughput sequencing technology. To gain insights into the changes in TILs induced by anti-CTLA-4 treatment in tumor hosts that respond or do not respond to therapy we used our well-characterized 4T1 mouse model in which anti-CTLA-4 treatment is effective only when combined with RT. Results show distinct contributions of RT and anti-CTLA-4 to increasing the number and clonality of TILs, and changes in clonal representation that are unique to the combination. These data suggest that RT effectively releases endogenous tumor antigens that prime anti-tumor T cells, supporting the concept that it can be used as a mean to generate an in situ individualized vaccine. We are currently exploring this hypothesis in clinical trials testing the combination of RT and checkpoint inhibitors.

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COMBINATION THERAPY

K6

Novel combination therapies for BRAF-mutant melanoma

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The ability to inhibit the activity of the MAPK pathway in BRAF-mutant melanoma has led to profound improvements in clinical outcomes for patients with advanced BRAFV600-mutant melanoma. Notably the additional of a MEK-inhibitor to a type-I BRAF inhibitor results in greater inhibition of MAPK-signaling resulting in improved partial and complete response rates, and prolongation of progression free and overall survival without increasing toxicity in normal cells. Three phase 3 clinical trials have shown highly consistent clinical outcomes defining the combination of a BRAF and MEK-inhibitor as a new standard of care for advanced BRAFV600-mutant melanoma. However resistance to the combination therapy occurs in the vast majority of patients necessitating the development of further novel strategies to overcome resistance. Intriguingly early genomic analyses of tumor tissue from patients progressing on combined BRAF and MEK-inhibition suggest reactivation of the MAPK-signaling as one mechanism of resistance. One strategy to overcome this is to inhibit MAPK-signaling downstream at key signaling nodes such as the cell cycle regulator CDK4. Our preclinical studies show that adding the CDK4-inhibitor palbociclib to a BRAF inhibitor will prevent the emergence of resistance. An alternative strategy is to combine BRAF-inhibitors with therapies that target other mechanisms of disease control such as the host response or tumor metabolism. It is clear that therapeutic combinations either sequentially or concurrently, will continue to define new standards of care for patients with advanced BRAF-mutant melanoma.

K7

Strategies and designs for combination immune therapy

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Journal of Translational Medicine 2015, **13(Suppl 1):**K7

Background: Tumors employ multiple mechanisms to escape immune surveillance and thus hamper cancer immunotherapies. Even when immune responses are generated with tumor vaccines the anti-tumor therapeutic outcome is often not feasible due to tumor-mediated immune suppression. These inhibitory mechanisms involve co-inhibitory receptor-ligand interactions, such as PD-1/PD-L1, secretion of inhibitory molecules, such as TGFβ, IL-10, IDO, and recruitment of suppressive cells, such as regulatory T cells (Treg), myeloid derived suppressor cells (MDSC), etc. Thus, successful cancer immunotherapy requires not only induction and enhancement of effector immune response but also simultaneous targeting of suppressor arm of immune system.

Materials and methods: Therapeutic and immune efficacy of mono- and combinational immunotherapies were tested in E7 antigen expressing TC-1 mouse tumor model. Tumor growth, survival, as well as peripheral and tumor-infiltrating immune cell profiles after immunotherapy were assessed.

Results: We developed multiple immune corrective strategies to target various tumor-mediated immune inhibitory mechanisms that enhance anti-tumor immunity and restructure tumor microenvironment to allow effector cells to function potently. We evaluated the immune and therapeutic efficacy of multiple combinational therapies, including blocking and agonist antibodies to co-inhibitory/co-stimulatory molecules, such as PD-1, PD-L1, OX40, CTLA-4, GITR, inhibitors and neutralizing antibodies to inhibitory cytokines/molecules, such as IL-10, TGFβ, IDO, and small molecules for selective inhibition of Tregs. In addition to evaluation of anti-tumor efficacy we also investigated cellular and molecular mechanisms of action for these agents when combined with different vaccine formulations and explored the interactions between compounds within combinational immunotherapies in animal tumor models.

Conclusion: We are demonstrating the importance of treatment sequence and scheduling when multiple agents are combined which requires full understanding of mechanisms of action for each component and can lead to the successful translation of developed treatment into the clinic.

K8

Combining targeted and immunotherapy: BRAF inhibitor dabrafenib (D) ± the MEK inhibitor trametinib (T) in combination with ipilimumab (Ipi) for V600E/K mutation-positive unresectable or metastatic melanoma (MM)

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Introduction: Dabrafenib, trametinib, and Ipilimumab are each indicated for treatment of patients (pts) with metastatic melanoma (Dabrafenib +trametinib in *BRAF* V600 mutation–positive metastatic melanoma). Dabrafenib and trametinib can be safely combined and prolong progression-free survival compared with monotherapy. Combining Dabrafenib+trametinib with the CTLA-4 antibody Ipi has the potential to improve treatment outcomes, but the safety profile is unknown. A recent report suggested caution in combining the BRAF inhibitor vemurafenib (V) with Ipilimumab; V+Ipilimumab resulted in G3 elevations of ALT in 6/10 pts leading to study discontinuation (NEJM2013 368; 14). The present study was designed to characterize the safety of Dabrafenib±trametinib+Ipilimumab, select recommended phase 2 doses (RP2Ds), and report efficacy.

Methods: Pts with stage IIIc/IV *BRAF* V600E /K mutation–positive MM and ≤1 prior treatments are eligible. Dose escalation occurs in cohorts of 3-6 pts followed by expansion (≤30 pts) at the RP2D. At data cutoff (Nov 8, 2013), 10 pts were enrolled: 4 received D+Ipi (doublet), 2 received D only (withdrawn before Ipi treatment), and 4 received D+T+Ipi (triplet).

Results: Median age of the 10 pts was 59.5 y (range, 32-75 y). **Doublet**: D 150 mg bid + Ipi 3 mg/kg q3w × 4 doses was well tolerated and selected as RP2D. No G3/4 ALT elevations or dose-limiting toxicities (DLTs) were observed. The most frequent adverse events (AEs; ≥2) were chills, fatigue, hand-foot syndrome, pyrexia, and maculopapular rash. Of 4 pts, 2 are ongoing and 2 stopped treatment (disease progression). Pts are currently being enrolled at this dose level in the expansion. **Triplet**: At current doses (D 100 mg bid/T 1 mg qd+Ipi 3 mg/kg q3w × 4), 2 out of 7 patients developed G3 colitis complicated by perforation.The triplet combination enrollment was therefore stopped. The most frequent AEs (≥2) were pyrexia, chills, arthralgia, insomnia, and maculopapular rash. One pt had G4 renal insufficiency that reversed rapidly.
Conclusions: To date the combinations of D+Ipi and D+T+Ipi appear to be tolerable and have not been associated with significant hepatotoxicity in MM, suggesting differences between BRAF inhibitors when combined with Ipi. However, combination of dabrafenib+trametinib+Ipilimumab was stopped early after 2 out of 7 patients developed colon perforation soon after initiating Ipilimumab therapy.

K9

MEK + CDK4 a regimen for non-BRAF V6000 melanoma

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Targeting of the $BRAF^{V600}$ mutation has proven a highly effective treatment strategy for the subset of melanoma carrying this mutation. However, the majority (~60%) of patients with advanced melanoma do not carry this mutation and are not targetable by selective BRAF inhibitors. The rapidly evolving genetic landscape of melanoma has revealed that the vast majority of *non-V600 BRAF* mutant melanoma still express other genomic alterations that lead to MAP kinase pathway activation. The most frequent of these are activating mutations in the *NRAS* gene representing 1/3 of the *non-V600 BRAF* mutant melanoma or 15-20% overall. The other MAPkinase activating genetic alteration include *NF1* loss of function, *MAP2K1*, *C-RAF*, *K-* or *H-RAS*, and alternate -non-V600 mutations in the *BRAF* gene. Therefore MEK inhibitors, ERK inhibitors, or even non-selective RAF inhibitors may represent effective therapeutic interventions. Early results in the NRAS mutant and non-*NRAS* or *BRAF V600E* mutant melanoma demonstrate clinical activity but likely not as robust as hoped. A trial with a MEK inhibitor (binmetinib) has demonstrated an unconfirmed objective response rate of approximately 20%. MEK inhibitors are also active against non-*NRAS*, *non-BRAF V600* mutant melanoma including responses in those melanomas carrying mutations in BRAF gene outside of V600 codon.
Genomic findings also demonstrate a high frequency of dysregulation of cell cycle regulatory proteins including genomic alterations in *CDKN2A*, *CDK4*, *Cyclin D1*, etc.
This finding suggests a role for CDK4/6 inhibitors in the therapy of melanoma. Additionally, preclinical work has demonstrated the effectiveness of the combination of MEK inhibitors (trametinib, binimetinib) with CDK4/6 inhibitors (palbociclib, LEE011) in multiple mouse models of *NRAS* mutant melanoma. New, selective, and more effective CDK4/6 inhibitors have arrived in the clinic including palbociclib and LEE011 to name several, Based on this background clinical investigations have recently begun in non-BRAF V600 mutant melanomas with combination MEK inhibitor and CDK4/6 inhibitor treatment. While very early in their development, Binimetinib and LEE011 has already produced objective clinical responses in about 1/3 of NRAS mutant melanoma patients with another 1/3 demonstrating clinical responses not meeting RECIST 1.1 criteria. Most impressive is the rapid nature of these responses and in some cases accompanying relief from tumor-related symptoms. Early on some significant toxicities have been observed and alternate schedules are being explored. These regimens are also being evaluated in the non-NRAS mutant, non BRAF V600 mutant melanomas. As premature as these results are they still provide excitement that targeting mutations other than $BRAF^{V600}$ is feasible and may provide hope for additional therapeutic options for the majority of melanoma patients.

K10

Combining radiation therapy with immunotherapy: clinical translation

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Ionizing radiation induces immunogenic cell death of tumors, an effect likely to contribute to the success associated with radiotherapy of cancer [1]. Recent discovery suggests that radiotherapy can be applied as a powerful adjuvant to immunotherapy and, in fact, can contribute to convert the irradiated tumor into an *in situ* vaccine, resulting in specific immunity against metastases [2]. Preclinical models of syngeneic tumors have reliably predicted clinical success, in distinct tumor settings and immunotherapy/radiation combinations [3-5]. As a first proof of principle trial, we translated the preclinical evidence of a successful combination with Flt3 ligand and RT [6] to a protocol of GM-CSF and RT, and demonstrated out of field objective responses in 27% of patients with multiple metastases of solid tumors, defined as an abscopal effect [7]. Parallel mechanistic studies in the lab in the syngeneic 4T1 mouse model of metastatic breast cancer demonstrated at intratravital microscopy that RT with anti-CTLA-4 increased the arrest of T cells in contact with tumor cells. The latter required interaction of NKG2D on CD8+ T cells with its ligand retinoic acid early inducible-1 (Rae-1) on the tumor cells, up-regulated by RT. Blocking NKG2D-Rae-1 interactions increased markedly the motility of anti-CTLA-4 treated T cells inhibiting their contact with irradiated tumor cells, and abrogated immune-mediated tumor rejection, suggesting a critical role of radiation-induced NKG2D ligands for the antitumor effects of anti-CTLA-4 [8]. In humans, a similar block of NKG2D is mediated by soluble MICA (sMICA), which is released by some tumors and reaches high levels in the serum [9]. Dranoff *et al* reported that in some patients sMICA levels dropped after initiation of Ipilimumab, due to the generation of anti-sMICA antibodies that led to its clearance [10]. Decreased levels of sMICA were associated with increased expression of NKG2D in T and NK cells, and corresponded to response to treatment. Anti-sMICA antibodies and sMICA levels can be measured in serum with ELISA by using recombinant MICA protein and anti-human MICA monoclonal antibodies [10]. Since RT is known to upregulate MICA on the surface of tumor cells [11] biopsies of tumors before and after radiotherapy and Ipilimumab could also be tested for expression of MICA.The preclinical success of the combination of anti-CTLA-4 antibody and RT was mirrored by abscopal responses in metastatic melanoma and NSCLC patients irradiated to one lesion, during Ipilimumab. This evidence inspired our current trial testing radiotherapy with CTLA-4 blockade in metastatic melanoma. In this study patients with newly diagnosed metastatic melanoma eligible to first line Ipilimumab are randomly assigned to Ipilimumab alone or Ipilimumab and radiotherapy to one metastatic lesion. Preliminary results in seven patients demonstrate feasibility of the combination, without additive toxicity. The novel role of radiotherapy as a powerful adjuvant to immunotherapy warrants more research to define the optimal immunotherapy/RT combinations: currently 35 trials of RT+immunotherapy are ongoing in USA.

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K11

Surgery in the combination therapies era

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Stage IV melanoma has historically had a poor prognosis because of lack of responsiveness to traditional chemotherapeutics. The 1-year survival rate of patients diagnosed with stage IV melanoma varies between 33 and 66 %. Depending on the number, the location, and the resectability of distant metastases, common treatment options comprise surgery, systemic medical therapy, and radiotherapy.
Single-agent DTIC has been the standard chemotherapy regimen for metastatic melanoma since 1970s. From a recent meta-analysis Luo et al [1] demonstrated that the substantial benefit from any of the various combinations of chemotherapy and biochemotherapy is about 4.2% with a mean overall survival of 6 months.
Patients who have limited sites of metastatic disease, a long disease-free survival and a tumor doubling time higher than 60 days, may be amenable to surgical resection and complete surgical excision of limited metastatic disease can result in prolonged relapse-free survival in carefully selected patients. The potential for complete surgical resection to benefit individual patients is suggested by the results of a prospective, phase II study from the Southwest Oncology Group. In this prospective, multicenter study, 64 of 77 carefully selected patients were able to undergo complete resection of all sites of metastatic disease. Overall, the three and four-year survival rates were 36 and 31 percent, respectively, although late relapses continued to be observed after this time.
Ipilimumab is a fully human monoclonal antibody directed against cytotoxic T-lymphocyteassociated antigen-4 (CTLA-4), a negative regulator of T-cell-mediated immune responses. In Phase III trials, ipilimumab treatment significantly extended overall survival (OS) compared with control in both pretreated and treatment-naïve patients. Approximately 10% of patients present objective responses by standard criteria, whereas 10–20% have stable disease or minor responses, that translate into a clinical benefit. In patients who initially achieve clinical benefit and then relapse months or years later, re-treatment with ipilimumab can be also advantageous.
Vemurafenib stands as the first personalized treatment in melanoma based on a specific mutation, with a favorable impact on survival. The overall survival rate at 6 months was 77% and 58% at 12.
As a consequence, future improvements of this targeted and personalized approaches are expected from ongoing clinical trials aiming at potentiate the activity of BRAF inhibitors through combination with other molecules. These combination therapies also aim at lowering the observed skin toxicities.

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NEWS IN IMMUMNOTHERAPY

K12

Grp94-specific monoclonal antibody to counteract BRAF inhibitor resistance in $BRAF^{V600E}$ melanoma

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The development of BRAF-I resistance in $BRAF^{V600E}$ melanoma underlies the need to develop strategies to counteract this resistance. It has been shown that administration of heat shock protein 90 (HSP90) inhibitors can counteract multiple mechanisms which drive BRAF-I resistance by reactivation of MAPK and activation of PI3K/AKT pathway. However the clinical application of this strategy is hampered the high toxicity associated with administration of currently available HSP90 inhibitors. To overcome this limitation we have developed a novel monoclonal antibody (mAb), named W9, which recognizes an extracellular epitope of glucose regulated protein of 94 kDa (Grp94), a member of the HSP90 family. The mAb W9 defined Grp94-epitope is selectively expressed on malignant cells but is not detectable on normal cells. Therefore targeting Grp94-epitope by mAb W9 is expected to cause limited if any side effects. The mAb W9 was found to increase and restore the sensitivity to BRAF-I of $BRAF^{V600E}$ melanoma cells including cells which have acquired BRAF-I resistance because of PDGFRα upregulation.

K14

Targeting multiple inhibitory receptors to reverse melanoma-induced T cell dysfunction

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It is now clearly established that dysfunctional/exhausted TA-specific T cells present in peripheral blood and at tumor sites co-express multiple inhibitory receptors. The implications of this important finding are two-fold. First, multiple subsets of TA-specific T cells can be identified in patients with advanced melanoma that exhibit variable levels of T cell dysfunction. Second, this observation supports the implementation of combinatorial therapies aiming at blocking multiple inhibitory pathways to enhance TA-specific immune responses and reverse tumor-induced T cell dysfunction. We have shown that a subset of highly dysfunctional TA-specific CD8+ T cells isolated from patients with advanced melanoma upregulate both PD-1 and Tim-3. PD-1 and Tim-3 blockades strongly enhance TA-specific CD8+ T cell expansion and function. Accordingly, targeting PD-1 and Tim-3 in *vivo* induces melanoma regression in mice. Therefore, the combination of PD-1 and Tim-3 blockade either alone or in combination with cancer vaccines appears to be a promising potent approach to reverse melanoma-induced T cell dysfunction and promote tumor regression in patients with advanced melanoma.

K15

Role of signal transduction and microRNAs on the immunogenicity of melanoma cells

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Melanoma tumors are heterogeneous and involve different processes generating cells with variable metastatic capacity, which also results in distinct clinical outcome of patients and variations in therapy responses. This might be due to different strategies leading to evasion of T and or NK

cell-mediated surveillance, which is accompanied by disease progression and a poor survival of melanoma patients. Therefore, the characterization of immune escape mechanisms might contribute to a better understanding of the development of the aggressive melanoma phenotype. This study analysed the underlying molecular mechanisms of HLA class I abnormalities and aberrant HLA-G expression in melanoma. A reduced mRNA and/or protein expression of various components of the MHC class I antigen processing machinery (APM) was identified in a large series of melanoma cell lines and lesions, which could be correlated with an increased tumor grading. In addition, adoptive T cell therapy also caused an escape from immune cell recognition by down-regulation components of the antigen processing machinery (APM). Recently, microRNAs (miRs) targeting HLA class I APM components were identified suggesting an important role for posttranscriptional control in this process. These miRs have functional activities on APM components, thereby also affecting HLA class I surface expression. Thus, these miRs might be used as novel targets for the treatment of melanoma or for selection of melanoma patients undergoing the most effective (immuno)therapy. In addition, a direct link of the IFN signalling pathway and the constitutive HLA class I APM component expression was found in melanoma. Down-regulation or loss of single components of the IFN-γ signal cascade was detected, which could be due to structural alterations, epigenetic mechanisms, such as methylation and/or histone deacetylation, or transcriptional/post-transcriptional deregulation. This was accompanied by a heterogeneous response of melanoma cells to IFN-γ treatment. Alterations in the IFN-γ signalling pathway were directly associated with a down-regulation of constitutive HLA class I components as demonstrated in melanoma lesions as well as in melanoma cell lines. Using shRNA-mediated silencing a direct effect of STAT1, JAK1 and JAK2 on MHC class I surface expression was confirmed. These data may provide novel insights into the complex regulation of HLA class I expression in melanoma and gives a rational for excluding patients for specific immunotherapies. Furthermore, strategies will be developed for restoration of the HLA class I positive phenotype.

K16

Advances in adoptive cellular therapy of cancer

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Adoptive cellular therapy is becoming an important cancer therapy. Tumor infiltrating T cells and peripheral blood T cells engineered to express high affinity T cell receptors specific to tumor antigens have been effective in treating melanoma. T cells engineered to express chimeric antigen receptors are being used to treat cancers and hematologic malignancies. Clinical studies have found that the characteristics of cellular therapies effects clinical outcomes. There is considerable variability between production lots of adoptive cellular therapies. This variability affects product characteristics and clinical outcomes. Production variability is due to both donor and manufacturing factors. Donor genetics as well as donor age, gender, race and prior therapies may affect product characteristics and function. The complexity involved with manufacturing cellular therapies also contributes to variability in the final product. Manufacturing adoptive cell therapies may involve stimulation with antibodies, cytokines, growth factors or allogeneic cells; prolonged culture and expansion; and genetic engineering. Product characteristics can affect clinical outcomes. Several studies have found that adoptive cellular therapies that have greater *in vivo* persistence and proliferation are associated with better clinical outcomes; however, the specific product characteristics responsible for better or worse clinical outcomes are not well understood. To better understand the critical characteristics responsible for the clinical effectiveness of cellular therapies we and others have been studying the potency of cellular therapies. Specific phenotypes have been associated with better clinical outcomes. Animal models suggest that T memory stem cells have greater proliferative potential and persistence than effector T cells and are more effective for adoptive cellular therapy. We have found that high throughput molecular assays are useful at identifying markers that are useful at measuring cell potency. In conclusion, in order to improve the clinical effectiveness of cellular therapies it's important to better understand the biological functions that contribute to product potency and develop potency markers and assays to ensure that the highest quality cells are consistently produced.

TUMOR MICROENVIRONMENT AND BIOMARKERS

K17

Molecular and cellular basis for immunomodulation with monoclonal antibodies

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Immunotherapy with immunostimulatroy monoclonal antibodies is based on the ability of antibodies to act as antagonists of inhibitory receptors or to act as agonists of costimulatory receptors. Interference of ligand to receptor binding underlies the first function while crosslinking of receptors to enforce signaling involves the second mode of action. Signalling receptors on lymphocytes and antigen presenting cells are embedded in the plasma membrane and are functionally linked to adaptors whose function is conducive to intracelluar signaling events.
In the case of antibodies blocking CTLA-4 functions multiple mechanism of action take place: (i) efficient competition for costimulatory interactions of CD28 with the very same ligands (CD80 and CD86), (ii) functional expression on the surface of regulatory T cells that can be depleted by ADCC, (iii) prevention of the denudation by cooption of CD80 molecules from the surface of antigen presenting cells, a(iv) interference with putative negative signalling mediated by phosphatases. The restricted pattern of expression of CTLA-4 on Tregs and activated T cells is critical to understand its function and exploitation in immunotherapy.
PD-1 upon interaction with PD-L1 or PD-L2 drives to the immune synapse the tyrosine phosphatases SHP-1 and SHP-2. These phosphatases, that are recruited to the cytoplasmic tail of PD-1 through its phosphorylated ITIM motive, dephosphorylate critical tyrosine residues in signaling adaptors activated by the concerted action of the TCR and CD28. Disruption of signalling in the immune synapse is prevented by blocking antibodies that avoid the guidance of PD-1 moieties to the synapse guided by PD-L1. Avoiding disruption of productive immune synapses is probably the main mechanism of action of anti-PD-1 and anti-PD-L1 blocking mAb.
The costimulatory members of the TNFR family transduce costimulatory signals by recruitment of the adptors TRAF-1, -2, -5. Signal transduction enhances NF-κB and MAPK signaling routes. In this pathway K-63 polyubiquitination reactions are critical for early signaling and are regulated by specific deubiquitinases. Receptors signal depending on crosslinking by the agonist antibody. The pattern of expression of these receptors on the different subset resting or primed/activated T cells determines function.
Overall precise understanding of the pattern of distributions of the receptors on immune cells and the signaling events that these receptors prevent or elicit will be ultimately important not only to understand the function but also for both therapeutic manipulation and biomarker finding.

K18

Genetic heterogeneity and transcriptional plasticity drive resistance during immunotherapy and targeted therapy

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Cancer is caused by genetic, epigenetic and microenvironmental changes that facilitate the survival and proliferation of tumor cells and their ability to acquire invasive properties. The plasticity of human tumor cells generally replicates normal molecular processes occurring during development and tissue repair. In humans, cancer progression is also shaped by host immune responses that edit the final tumor-host

interactions. The genetic complexity and extreme variability of human melanoma means a multidisciplinary integrative approach is needed to understand the interactions between the genetic background of the host, the tumor and its microenvironment, and the impact of these on the immune system. It is becoming evident that successful anti-tumor strategies need to encompass a multimodal approach to avoid tumor escape or relapse, combining agents able to block essential signal transduction pathways with immunotherapy.

K19

The immune-related role of BRAF in melanoma

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Background: In the recent years there have been major advances in the field of cancer immunology and the existence of a dichotomy between immunologically active and quiescent tumor phenotypes has been recognized in several cancers. The activation of a Th1 immune signature has been shown to confer better prognosis and likelihood to respond to immunotherapy. However, whether such dichotomy depends on the genetic make-up of individual cancers is not known yet. In melanoma, BRAF and NRAS mutations are commonly acquired during tumor progression. Although the oncogenic potential of BRAF and NRAS alterations has been attributed to reduced apoptosis, increased invasiveness and increased metastatic behavior, the role of BRAF and NRAS in the immunological landscape of cutaneous melanoma has been poorly investigated and the effects of BRAF and NRAS mutations on global gene expression remain to be understood. We explored the role of BRAF and NRAS mutations at the transcriptome level and in influencing the immune phenotype (based on a classification previously identified by our group).

Materials and methods: One-hundred-thirteen pre-treatment snap frozen tumor biopsies were collected from patients treated at the Surgery Branch, NCI (Bethesda, Maryland) and processed for DNA and RNA isolation. Each sample underwent microarray analysis and BRAF and NRAS genotyping. Allele-specific PCR was also performed in order to exclude low-frequency mutations. Fifteen melanoma cell lines were also tested for BRAF and NRAS mutation by Sanger sequencing and RNA-sequencing.

Results: Comparison between BRAF and NRAS mutant versus wild type samples identified mostly constituents or regulators of MAPK and related pathways. Initially, we postulated that there might be a common MAPK activation signature resulting from either BRAF or NRAS mutation; however, we found no overabundance of discriminatory genes for the combined group of samples displaying either BRAF or NRAS mutations. This suggests that the transcriptional consequences resulting from mutations of BRAF or NRAS might be different, although there was overlapping of some genes, presumably due to their differential capacity to receive input signals and transduce them through different effectors.

When testing gene lists discriminative of BRAF, NRAS and MAPK alterations, we found that 112 BRAF-specific transcripts were able to distinguish the two immune-related phenotypes already described in melanoma, with the poor phenotype associated mostly with BRAF mutation. Noteworthy, such association was stronger in samples displaying low BRAF mRNA expression. However, when testing NRAS mutation, we were not able to find the same association. Class comparison between BRAF mutant samples with high and low expression of the same gene identified 6296 transcripts. Functional interpretation analysis showed that these 6296 transcripts were associated to IL-2 and JAK/Stat signaling pathways, supporting the immunoregulatory role of BRAF. Additionally, fifteen melanoma cell lines were also tested by BRAF and NRAS DNA genotyping and RNA-sequencing. Interestingly, we found that among 8 cell lines BRAF mutated (V600E), three of them expressed BRAF at low level and may have preferential wild type allele selection at the RNA level.

Conclusion: In conclusion we provide novel insights into the effect of BRAF and NRAS mutations on gene expression according to the immune classification. However, further deeper analyses are warranted to understand the mechanisms behind the association of BRAF mutation with a poor immune phenotype and also behind BRAF low expression and wild type allele selection at the RNA level.

K20

Is there a role for immune checkpoint blockade in metastatic uveal melanoma?

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Introduction: Uveal melanoma is the most common intraocular malignancy in adults with a disease specific mortality rate of ~40%. Oncogenic mutations in *GNAQ* and *GNA11* were recently identified as driver mutations in ~90% of uveal melanoma. While localized disease can be effectively treated by surgery or radiotherapy, treatment options for metastatic uveal melanoma are limited. To investigate the interplay between uveal melanoma and the hosts´ immune system and to test immunotherapeutic approaches, we have established an syngenic mouse model of *GNAQ* oncogene-driven melanoma.

Materials and methods: Melan-a cells were transduced with retroviruses expressing an activating Q209L mutation of *GNAQ*. Tumor formation was measured with calipers after subcutaneous inoculation of the cell line in C57BL/6 or $RAG2^{-/-}$ mice. Splenocytes were harvested at the time of sacrifice. For NK cells depletion, an anti-NK1.1 antibody was administered weekly. Murine anti-CTLA-4 antibodies (100µg), anti-PD-1 antibodies (250µg) or the combination of both were given weekly in a therapeutic setting. Peripheral blood mononuclear cells (PBMC) from uveal melanoma patients were obtained after informed consent and analyzed by flow cytometry.

Results: Melan-a cells with mutant but not with wt *GNAQ* form tumors when injected into mice, resulting in a model of G-protein-driven experimental murine melanoma (GEM). No significant difference in tumor growth was observed between C57BL/6 or $RAG2^{-/-}$ mouse strains. Elimination of NK cells in $RAG2^{-/-}$ mice led to increased tumor growth compared to non-depleted controls ($p<0.05$). Multi-color flow cytometry revealed a significant increase of $CD11b^{+}Gr\text{-}1^{int}$ myeloid-derived suppressor cells (MDSC) as well as regulatory T cells (Treg) in tumor-bearing animals as compared to non-tumor-bearing animals. In PBMC from patients with metastatic uveal melanoma, an increased frequency of monocytic MDSC and Treg was found as compared to healthy controls. In the GEM model, concurrent administration of an anti-CTLA-4 antibody and an anti-PD-1-antibody does delay tumor growth significantly ($p<0.05$) while monotherapy with either agent did not. No reduction in the accumulation of Treg and MDSC was found when comparing treated and untreated mice.

Conclusion: In the syngenic GEM model, tumor growth seems to be controlled by NK cells and can be delayed by dual immune checkpoint blockage. In this model, tumor formation is accompanied by an immunosuppressive leukocyte network also found in patients with metastatic uveal melanoma. Specific elimination of these cells might therefore synergize with immune checkpoint blocking antibodies. This hypothesis needs to be tested in future studies to better define its treatment potential for patients with advanced uveal melanoma.

ORAL PRESENTATIONS

MOLECULAR AND IMMUNO ADVANCES

O1

Cytokine Induced Killer cells effectively kill chemo-resistant melanoma cancer stem cells

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Background: Metastatic Melanoma (mMel) is considered refractory to conventional chemotherapies. New molecular targeted approaches, inhibiting mutated forms of the serine-threonine kinase B-RAF, significantly increased the response rate but patients almost invariably relapse and prognosis remains severe [1,2]. Open challenge is the characterization and targeting of cancer stem cells (CSCs), considered responsible for chemoresistance and disease relapse. Adoptive immunotherapy holds great promises for the treatment of mMel and research efforts are ongoing to explore its potential activity against melanoma CSCs (mCSCs). Cytokine Induced Killer (CIK) cells are a subset of *ex vivo* expanded T lymphocytes with mixed $CD3^+CD56^+$ phenotype and endowed with HLA-unrestricted tumor killing activity. We and others recently reported the preclinical activity of CIK cells against several solid tumors including mMel [3].
Aim of our research is to explore the preclinical activity of CIK cells against autologous mCSCs surviving treatments with chemo or molecular targeted therapies.
Material and methods: We set a preclinical autologous immunotherapy model with primary melanoma cultures and CIK cells generated from patients treated at our Center. To visualize mCSCs we transduced tumor cells with a lentiviral CSC-detector vector encoding enhanced Green Fluorescent Protein (eGFP) under control of the stem-gene oct4 promoter. We treated all mMel cultures with chemotherapy drug fotemustine (IC50 dose). Melanoma cultures harboring BRAF V600E mutations were also treated with BRAF inhibitor (BRAFi) dabrafenib (IC50 dose). We evaluated the presence of residual mCSCs among mMel cells surviving 72h of treatment and explored their susceptibility to immunotherapy with autologous CIK cells.
Results: We visualized mCSCs within 11 mMel cultures (3/11 BRAF V600E mutated); median value of eGFP+mCSCs was 15% (range 3.5-26.4%). Putative mCSCs displayed a relative resistance to conventional treatments. The presence of eGFP+mCSCs increased of 39% and 25% among melanoma cells that survived exposure to fotemustine and BRAFi respectively, compared to untreated controls (n=11). CIK cells effectively killed autologous mMel and mCSCs. Tumor specific killing, equally involving bulk mMel targets and mCSCs, ranged between 75% and 33% at effector target ratios of 40:1 and 1:1 respectively (n=8).
Conclusion: We provided first proof of concept that putative mCSCs are relatively resistant to conventional treatments with chemo or molecular targeted therapy and susceptible to immunotherapy killing by autologous CIK cells. These preclinical findings support the hypothesis that mCSC may be responsible for disease relapses and support designing of experimental immunotherapy clinical trials with CIK cells in mMel settings.
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O2

RSK1 activation promotes invasion in nodular melanoma

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Background: The two major melanoma histologic subtypes, superficial spreading and nodular melanomas, are believed to differ in their speed of dermal invasion but to converge biologically once they invade and metastasize. Here, we tested the hypothesis that distinct molecular alterations arising in primary melanoma cells might persist as these tumors progress to invasion and metastasis.
Materials and methods: Expression of 141 signaling proteins was evaluated by protein pathway array in 3 Radial Growth Phase (RGP)/SSM and 3 Vertical Growth Phase (VGP)/NM cell lines. The impact of p90-ribosomal-S6-kinase (RSK1) and its inhibition on proliferation, migration and invasion was assessed in SSM and NM cell lines, and confirmed using NM cells treated with a RSK inhibitor (BI-D1870) in microarray profiling studies. The effect of constitutive RSK1 activation in vivo was further studied using a zebrafish model.
Results: We show that p90-ribosomal-S6-kinase (RSK1) was significantly hyper-activated in human melanoma lines and metastatic tissues derived from nodular compared with superficial spreading melanoma. RSK1 was constitutively phosphorylated at Ser-380 in nodular but not superficial spreading melanoma and was not directly correlated with BRAF or MEK activation. Nodular melanoma cells were more sensitive to RSK1 inhibition using both siRNA and pharmacological inhibitor BI-D1870 compared with superficial spreading cells. In addition, gene expression microarray analyses revealed that RSK1 orchestrates a program of gene expression that promotes cell motility and invasion. Our data also demonstrate a differential overexpression of the pro- metastatic MMP-8 and TIMP-1 in metastatic nodular compared to metastatic superficial spreading melanoma. Finally, using an in vivo zebrafish model, constitutive RSK1 activation increased melanoma invasion.
Conclusions: Together, our data reveal a novel role for activated RSK1 in the progression of nodular melanoma, and suggest that melanoma originating from different histological subtypes may be biologically distinct and that these differences are maintained as the tumors invade and metastasize.

COMBINATION THERAPY

O3

Long-term overall survival from a phase I trial using intratumoral plasmid interleukin-12 with electroporation in patients with melanoma

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Journal of Translational Medicine 2015, **13(Suppl 1):**O3

Background: In 2007, a phase 1 dose-escalation safety study of intratumoral electroporation (EP) of plasmid interleukin-12 (pIL-12) was completed in 24 patients (pts) with malignant melanoma. IL-12 is thought to promote interferon-γ-pathway activation and antigen presentation and processing machinery (APM) to enhance systemic anti-tumor response. This phase 1 study was the first ever gene therapy in humans to use EP to locally deliver DNA plasmid to induce systemic anti-tumor immunity. Here we present long-term overall survival (OS) results from this study.
Methods: Twenty-four pts with stage IIIB-IV melanoma and with at least two accessible lesions received one treatment cycle of pIL-12 EP administered on days 1, 5 and 8. Dose escalation with increasing plasmid concentrations was performed in cohorts 1 through 5. Plasmid was dispensed at concentrations of 0.1, 0.25, 0.5, 1.0 and 1.6 mg/mL, and plasmid volume was calculated based on tumor volume. Two additional cohorts were enrolled (cohorts 6 and 7) and received a total dose of 3.8 or 5.8 mg/treatment respectively, divided among two to four tumor sites, irrespective of tumor volume. Tumor responses were evaluated by modified RECIST. Biopsies were obtained for tumor histology, including evaluation of lymphocytic infiltrate, and tissue intratumoral IL-12 concentration.
Results: In this phase 1 study, 53% (10/19) pts with metastatic disease had a systemic response to the local pIL-12 EP treatment as evidenced by stable disease or objective regression of non-injected lesions. Additionally, 11% (2/19) experienced complete regression of all distant lesions without concurrent or subsequent systemic therapy. The most frequent adverse event (AE) related to treatment was transient pain during the EP procedure, with 54% (13/24) pts reporting grade 1 and 46% (11/24) pts reporting grade 2 pain. No DLT was noted within any of the treatment cohorts. The median OS (n=24, ITT) was 24.2 mos. In pts who experienced SD or better with pIL-12 EP (n=9), OS of 46.4 mos was observed, a 32.9 mos increase compared to non-responders (OS 13.5 mos; n=15).
Conclusions: Results from this phase 1 study demonstrate that local treatment with pIL-12 EP successfully induces systemic anti-tumor immune-mediated effects without severe local or systemic toxicity. Moreover, long-term follow-up data suggest that systemic disease stabilization with pIL-12 EP is correlated with improved survival. Based on this evidence, intratumoral EP of pIL-12 is an effective tool for gene transfer of DNA plasmid with potential applications as both a monotherapy and in combination with other agents that promote anti-tumor immunity.

O4

coBRIM: a phase 3, double-blind, placebo-controlled study of vemurafenib versus vemurafenib + cobimetinib in previously untreated *BRAF*^{V600} mutation–positive patients with unresectable locally advanced or metastatic melanoma (NCT01689519)

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Background: Combined inhibition of BRAF and MEK is hypothesized to improve clinical outcomes by preventing or delaying onset of resistance observed with BRAF inhibitors alone. This randomized phase 3 study evaluated the combination of the BRAF inhibitor vemurafenib and the MEK inhibitor cobimetinib.
Materials and methods: 495 patients were randomly assigned to receive vemurafenib + cobimetinib (60 mg QD, 21 days on/7 days off) or vemurafenib (960 mg BID) + placebo. Eligibility included treatment-naive *BRAF*^{V600} mutation–positive patients with unresectable locally advanced or metastatic melanoma and adequate performance status and organ function. The primary end point was investigator-assessed progression-free survival (PFS). Safety monitoring included serial cardiac and ophthalmic evaluation and measurement of creatine phosphokinase.
Results: Median PFS was 9.9 months with the combination compared with 6.2 months with the control (HR, 0.51; 95% CI, 0.39-0.68; $P<0.0001$). Objective response rate (ORR) was 68% in the combination and 45% in the control arm ($P<0.0001$), including complete response in 10% in the combination and 4% of patients in the control group. Subgroup analyses of PFS based on key demographic and tumor characteristics were consistent with PFS in the intent-to-treat population, including those with normal or elevated baseline lactate dehydrogenase (LDH). PFS assessed by independent review was comparable with investigator-assessed PFS. Interim overall survival (OS) data showed an HR of 0.65 (95% CI, 0.42-1.00) but did not cross the prespecified stopping boundary. Compared with vemurafenib alone, the combination was associated with a higher incidence of grade 3 or 4 adverse events (65% vs 59%), with no difference in the rate of adverse events leading to study drug discontinuation (13% vs 12%). Most grade ≥3 events occurred in the first 28 days and resolved quickly. Known MEK inhibitor–related toxicities such as diarrhea, serous retinopathy, elevated creatine phosphokinase, and increased liver transaminase levels were more commonly observed with the combination. The majority was grade 1 or 2, occurred between 1 and 4 months in the treatment course, and resolved quickly. The occurrence of secondary cutaneous neoplasms decreased with the combination (4% vs 18%). Photosensitivity was more common in patients treated with the combination (all grades 32% vs 18%).
Conclusion: Cobimetinib + vemurafenib significantly improved PFS and response rate among patients with *BRAF*^{V600}-mutant metastatic melanoma, with promising preliminary OS analysis. Most combination-related toxicities are mild or moderate, occur early in treatment, and are manageable by dose modification and supportive care; treatment discontinuation is uncommon.
Clinical trial registration number: NCT01689519.

NEWS IN IMMUMNOTHERAPY

O5

A randomized controlled comparison of pembrolizumab and chemotherapy in patients with ipilimumab-refractory melanoma

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Background: Pembrolizumab blocks the interaction between PD-1 and its ligands PD-L1 and PD-L2, thereby inducing an antitumor immune response. In a phase I study, pembrolizumab demonstrated promising antitumor activity and acceptable safety in patients with ipilimumab-treated melanoma, leading to accelerated approval in the US.
Materials and methods: KEYNOTE-002 is a randomized phase 2 study in patients with ipilimumab-refractory melanoma (ie, confirmed PD in the 24 weeks following ≥2 ipilimumab doses) and, if *BRAF* mutant, previously treated with a BRAF inhibitor. Patients were randomized 1:1:1 to pembrolizumab 2 or 10 mg/kg Q3W or investigator-choice chemotherapy (carboplatin + paclitaxel, carboplatin, paclitaxel, dacarbazine, or temozolomide). Patients with PD confirmed by independent central review could cross over to pembrolizumab treatment after the first 3-month assessment. Primary objective of the interim analysis prespecified to occur after ≥ 270 PFS events (RECIST v1.1, independent central review) was to evaluate the superiority of either pembrolizumab dose over control for PFS at a 1-sided 0.25% significance level (estimated HR 0.66).
Results: From Nov 2012 to Nov 2013, 540 patients from 12 countries enrolled. Based on central review of a total of 410 PFS events, the HR was 0.57 and 0.50 for pembrolizumab 2 and 10 mg/kg Q3W, respectively, over control (P<0.00001 for both comparisons). The 6-month PFS rate was 34% (95% CI 27%-41%) and 38% (95% CI 31%-45%) for pembrolizumab 2 and 10 mg/kg, respectively, compared with 16% (95% CI 10%-22%) for the control arm. PFS by investigator assessment was similar to that of central review. The PFS effect was consistent in all subgroups. ORR was 21% at 2 mg/kg, 25% at 10 mg/kg, and 4% in the control arm (P<0.0001 for both comparisons). Median response duration was not reached in either pembrolizumab arm and was 37 weeks in the control arm. Forty-eight percent of patients in the control arm crossed over to pembrolizumab treatment. OS data are not mature (final OS analysis will be performed after 370 deaths have occurred). The safety profile was consistent with that previously observed for pembrolizumab. Despite a decreased therapy duration, rates of grade 3-5 drug-related AEs were numerically higher in the chemotherapy control arm (26%) than in the pembrolizumab 2-mg/kg (11%) and 10-mg/kg (14%) arms.
Conclusion: Both pembrolizumab doses met prespecified criteria for PFS superiority over the chemotherapy control arm. Pembrolizumab significantly prolongs PFS compared with chemotherapy, approximately doubling the 6-month rate in an ipilimumab-refractory population.
Clinical Trial Registration Number: NCT01704287.

O6

Nivolumab improved survival vs dacarbazine in patients with untreated advanced melanoma

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Journal of Translational Medicine 2015, **13(Suppl 1):**O6

Background: The phase 1 study of nivolumab, a fully human IgG4 programmed death-1 (PD-1) immune checkpoint inhibitor monoclonal antibody, showed promising antitumor activity in patients with advanced melanoma.
Materials and methods: This phase 3 study compared nivolumab vs dacarbazine in treatment-naïve patients with BRAF wild-type metastatic melanoma. Patients were randomized 1:1 to receive nivolumab 3 mg/kg every 2 weeks (Q2W) + placebo Q3W (n = 210) or dacarbazine 1000 mg/m2 Q3W + placebo Q2W (n = 208) until disease progression or unacceptable toxicity. Randomization was stratified by M-stage and programmed death ligand-1 (PD-L1) status. The primary endpoint was overall survival (OS). Patients were followed for up to 16.7 months at the time of data cutoff, which occurred 5.2 months after the first visit of the last patient randomized.
Results: The hazard ratio (HR) for death was 0.42 (99.79% CI 0.25–0.73; P < 0.0001) in favor of nivolumab, with 1-year OS rate 73% (95% CI, 66%–79%) for nivolumab vs 42% (95% CI, 33%–51%) for dacarbazine. Median OS was not reached for nivolumab and was 10.8 months for dacarbazine. Median progression-free survival (PFS) was 5.1 months for nivolumab and 2.2 months for dacarbazine (HR for death or progression 0.43, 95% CI 0.34–0.56; P < 0.0001). Objective response rate was 40% (84/210) vs 14% (29/208) for nivolumab and dacarbazine, respectively (P < 0.0001). Median duration of response was not reached for nivolumab and 6 months for dacarbazine. At the time of data cutoff, responses were ongoing in 86% (72/84) of nivolumab and 52% (15/29) of dacarbazine responders. PD-L1 positivity (using a 5% tumor cell surface staining cutoff) appeared to be associated with improved OS in the nivolumab arm (85% of PD-L1+ and 71% of PD-L1-/indeterminate patients alive at the time of last follow-up). Both PD-L1+ and PD-L1-/indeterminate patients receiving nivolumab had improved OS vs dacarbazine (un-stratified HR 0.30, 95% CI, 0.15–0.60 in PD-L1+ patients; 0.48, 95% CI, 0.32–0.71 in PD-L1-/indeterminate patient, both in favor of the nivolumab arm). The most common nivolumab-related adverse events (AEs) were fatigue, pruritus, and nausea. Drug-related grade 3–4 AEs were reported in 12% vs 18% of patients receiving nivolumab vs dacarbazine, respectively. AEs led to discontinuation in 7% and 12% of dacarbazine- vs nivolumab-treatment patients, respectively.
Conclusions: Compared to dacarbazine, nivolumab significantly improved OS and PFS in previously untreated patients with BRAF wild-type metastatic melanoma with an acceptable safety profile.
Clinical Trial Registration Number: NCT01721772.

O7

Ipilimumab treatment decreases circulating Tregs and GrMDSC while enhancing CD4+ T cell activation

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Background: Ipilimumab 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. There is a need for well-documented

pharmacodynamic markers together with potential predictive biomarkers that may allow for pretreatment selection of patients and screening for IRAE. Most of the recently published immune monitoring studies focus mainly on the effect that ipilimumab has on T cell populations. An in-depth immune monitoring study was conducted in advanced melanoma patients undergoing treatment with ipilimumab. The main focus of this work was to analyze the effect of ipilimumab treatment on peripheral blood MDSC populations as well as T cells.

Materials and methods: Six patients with advanced stage melanoma received ipilimumab treatment at 3 or 10 mg/kg doses as part of double blind randomized trial CA184-169. Twenty-four additional patients received 3 mg/kg doses. Blood samples were collected before treatment (baseline) and at the time of the second and fourth ipilimumab doses. Peripheral blood mononuclear cells were isolated by density gradient centrifugation and stained for flow cytometric analysis within two hours of sample collection.

Results: In the thirty patients included, median OS was 38 weeks from the start of treatment. Adverse events were observed in 12 patients (40%), including seven grade III-IV (23%). Patients were classified according to their response as PD (progressive disease, 57%), SD (stable disease, 23%) and PR (partial response, 20%).

$ICOS^+CD4^+$ T cell frequency was significantly increased after Ipilimumab treatment, suggesting an increase in the activation of this cellular population. The endpoint mean frequency of Tregs was significantly lower than the baseline. In addition to this, changes in the suppressive myeloid compartment were also observed, with significant reductions in the frequency of granulocytic MDSCs. The suppressive potential of both granulocytic and monocytic MDSCs was also diminished, as the frequencies of $Arg1^+$ and $iNOS^+$ cells were reduced after treatment. Further analysis showed that the populations of granulocytic MDSCs and $CD4^+ICOS^+$ T cell populations presented a statistically significant correlation.

Conclusions: Ipilimumab treatment can be acting in two very distinct ways: on one hand it is increasing the activation status of T cells and on the other hand, it is decreasing the frequencies of suppressive cells such as Tregs and granulocytic MDSCs. These effects, and their possible correlations to clinical benefit, should be further explored.

O8

Analysis of T and NK cells immune response in Ipilimumab treated Melanoma patients

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Background: The most promising immunotherapeutics tested in metastatic melanoma patients are the monoclonal antibody blocking CTLA-4 (Ipilimumab), and those interfering with PD-1 and PD-L1. However, the lack of knowledge on predictive biomarkers that could assist the treatment remains a limiting factor. We speculate that, along with additional markers, the immunoscore [1] is fundamental as prognostic and predictive marker for response to immunotherapies in metastatic melanoma. Our previous data demonstrate that NK cells control the melanoma progression [2,3]. Therefore we have analysed both T cells and NK cells subsets frequencies and receptors repertoire in the peripheral blood of Ipilimumab treated patients with Stage IV metastatic melanoma.

Material and methods: Peripheral blood mononuclear cells (PBMCs) from 12 different patients with stage IV metastatic melanoma were collected and analyzed. Each patient received 4 infusions of Ipilimumab each 21 days. Before each infusion we collected patients' blood and isolated PBMCs to analyze the lymphocytes compartment.

We stained for the following antibodies: CD56 PE, CD3 Fitc, CD56 APC, CD4 PeCy7, CD8 APCCy7, CD152 (CTLA4) PE, CD279 (PD-1) PE, CCR7 PeCy7, CD158a/h(KIR2DL1/S1) PE, CD158b (KIR2DL2/DL3) PE, CD158e (KIR3DL1) PE, CD16 APCCy7, CD57 PE, CD69 PE, CD314 (NKG2D) PE, CD226 (DNAM-1) PE, CD337 (NKp30) PE, CD336 (NKp44) PE, CD335 (NKp46) PE (Miltenyi Biotech), CD192 (CCR2) AlexaFluor 647, CXCR2 (IL8RB) APC, 7-AADStaining Solution (BD Italia), TIM3 PE (ebioscience), NKG2C PE (R&D Systems). The analysis was performed with FACS CANTO II. Statistical analysis was performed with Anova and Student's t-test.

Results: Our data indicate that, after the first Ipilimumab treatment, an inversion of CD4/CD8 ratio occurs with a concomitant increase in the $CD56^{dim}$ population and a higher expression of TIM-3 and NKp46 molecules on the surface of NK cells. Moreover, the frequency of NK and T cells expressing KIRs and CCR7 is reduced, while the mean fluorescence intensity of CD16 and PD1 is upregulated on both $CD56^{bright}$ and $CD56^{dim}$ NK cells.

Conclusions: These preliminary data indicate that early during Ipilimumab treatment, cytotoxic lymphocytes $CD8^+$ T cells and $CD56^{dim}$ NK cells expand and become activated. NK cells seem to be polarized towards a $CD56^{dim}CD16^{bright}$ $KIRs^+NKp46^+TIM3^+$ phenotype. Ipilimumab treatment may induce NK cells maturation, which might in turn drive activation of $CD8^+$ T cells.

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TUMOR MICROENVIRONMENT AND BIOMARKERS

O9

Tumor-infiltrating lymphocytes predict cutaneous melanoma survival

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Background: Tumor-infiltrating lymphocytes (TILs) is considered a manifestation of the host immune response to tumor, but the role of TILs on melanoma mortality is controversial. Therefore, the aim of this study was to investigate the role of TILs on melanoma mortality, controlling for all known histological prognostic parameters.

Materials and methods: We conducted a 10-year cohort study among 4143 patients from the same geographic area (Lazio) with primary cutaneous melanoma diagnosed between January 1998 and December 2008. Survival probability was determined by Kaplan–Meier estimates, and prognostic factors were evaluated by multivariate analysis (Cox proportional hazards model).

Results: Survival decreased with increasing age (P for trend < 0.001) and Breslow thickness (P for trend < 0.001). In the multivariate Cox model, the presence of high levels of tumour infiltrating immune cells in primary invasive melanomas was associated with lower risk of melanoma death (RR: 0.32; 95%CI:0.13-0.82, P for trend <0.001), after controlling for sex, age, breslow thickness, histological type, mitotic rate and ulceration.

Conclusions: These results suggest that immune microenvironment affects melanoma survival. Understanding differences in survival across distinct subgroups of melanoma patients may help choosing types of therapy.

O10

αβ-Double Negative CD4/CD8 (CD56) T cell (DNTs) in metastatic melanoma: basal frequency and behaviour during Ipilimumab treatment. Preliminary evaluations

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Journal of Translational Medicine 2015, **13(Suppl 1):**O10

Background: The knowledge of the immune system role on melanoma has accelerated the translation of key advancements into medical breakthroughs like ipilimumab, an anti-CTLA4 immunomodulating antibody. Ipilimumab works amazingly well only in a limited number of patients and its effects on T-cell subpopulations as well as on immune response remains to be elucidated. Recently, it was described a new subset of immunomodulating T-cells, known as Double-negative T-cells (DNTs) expressing either αβ or γδ T-cell receptors (TCR) but lacking CD4,CD8,CD56. The DNTs contribute specifically to anti-tumor immunity since involved in immune regulation and tolerance acting as regulatory T-cells (Treg) and/or cytotoxic T-cells and they contribute to *in vivo* anti-melanoma immunity as previously reported [1-5]. However no data are available on their frequency in melanoma, as well as the effects of ipilimimumab on DNTs functional attitude in immunomodulation and on modulating their expression during the therapy. We aimed to evaluate the modulation of DNT frequency in Metastatic Melanoma (MM) patients treated with ipilimumab during the therapy in order to explore their potential role on clinical outcome and therapy response.

Patients and methods: We carried out flow cytometric studies and statistical analyses on data of frequency of DNTs from 136 individuals, which included 16 healthy donors,30 MM patients who received ipilimumab as second line therapy, and 90 lymphoma patients. To evaluate the modulation of DNT during ipilimumab therapy we collected peripheral blood of MM subset at three time points: 1. Before start of therapy, 2. Before the 3th ipilimumab infusion, 3. After 2 months from the end of therapy. All patients provided their informed consent in accordance with the Declaration of Helsinki.

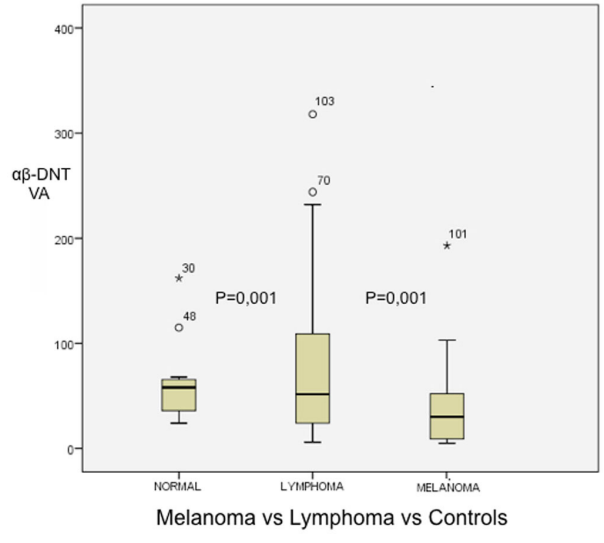

Fig 1(abstract O10) Circulating αβ-DNT frequency in Melanoma patients as compared with healthy controls and Lymphoma patients P=0,048

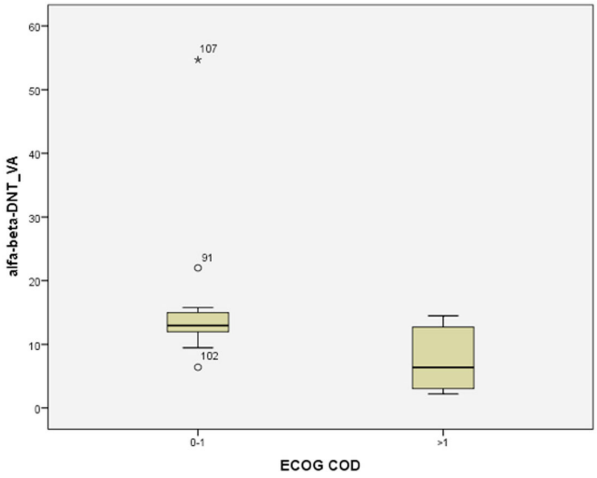

Fig 2(abstract O10) Circulating αβ-DNT frequency in Melanoma patients based of ECOG performance status

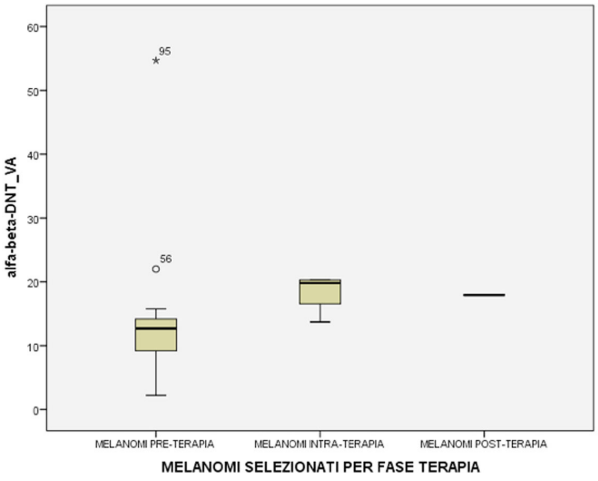

Fig 3(abstract O10) Circulating αβ-DNT frequency in Melanoma patients during the Ipilimumab treatment.

Results: We observed a significant decrease (p =0.001) of circulating αβ-DNT frequency in MM (13.49cells/ul±5.4) as compared with healthy controls (31.3cells/ul ± 3.4) and more interestingly when compared with Lymphoma patients (p=0.001) (fig.1). Furthermore, αβ-DNTs was significantly increased (p=0.048) in MM patients with ECOG performance status ≤1 as compared with >1 (fig.2). Finally, despite a low cluster was collected until the last time point sample, we observed a trend of significant increasing (p=0.083) in αβ-DNTs during the ipilimumab treatment (fig.3).

Conclusions: This is the first report on evaluation of αβ-DNTs in MM patients and their modulation by an immunomodulating drug such as ipilimumab. Our preliminary data suggested a lower frequency of αβ-DNTs and a worse immunological impairment in MM compared to healthy and lymphoma subject as well as a trend of increasing of this T-cell population during the therapy. These results, supported by future studies, could estabilish the role of DNTs in MM patients and the significance of their increase during ipilimumab therapy.

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O11

Intratumoral electroporation of plasmid interleukin-12: efficacy and biomarker analyses from a phase 2 study in melanoma (OMS-I100)

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Background: Recent data from immune checkpoint studies, including studies of anti-PD1 and anti-PDL1 antibodies, suggest that an inflammatory intratumoral milieu is required for an optimal response to immune therapy. Serial biopsy analyses from a phase 1 study demonstrate that transformation of tumor cells with electroporation (EP) of plasmid interleukin-12 (pIL-12) promotes this inflammatory immune milieu. Here we present clinical response data for 30 advanced melanoma patients (pts) treated with pIL-12 EP in a phase 2 trial (OMS-I100). We also present additional safety and more detailed biomarker data demonstrating the promotion of pro-inflammatory genes with pIL-12 EP therapy.
Methods: Thirty patients with stage IIIB-IV melanoma received up to 4 cycles of pIL-12 EP into superficial cutaneous, subcutaneous, and nodal lesions on days 1, 5 and 8 of each 12-week cycle. Tumor responses were evaluated using modified RECIST criteria for cutaneous lesions. Adverse events (AEs) were assessed using CTCAE version 4. Alterations in transcription were assessed by comparing pre- and post-treatment biopsies from treated lesions using NanostringTM technology to identify pharmacodynamic markers of downstream pathway activation and to characterize cellular infiltration.
Results: The best overall response rate (BORR) by modified RECIST in 29 evaluable pts was 31% (9/29), with 10% (3/29) of pts achieving a CR. Regression of at least one non-treated lesion was seen in 54% (13/24) of pts with evaluable lesions. The most common treatment-related adverse event (AE) reported was transient Grade 1/2 pain at the treatment site, reported in 87% (26/30) of pts. Grade 3 adverse events were rare and included only 1 report of Grade 3 pain at the injection site. No grade 4 or higher adverse events were observed. Analysis of tissue samples from patients treated with pIL-12 EP showed a gene expression pattern consistent with downstream activation of NK cells and interferon-γ-dependent genes, including key genes responsible for tumor inflammation, antigen processing and presentation (APM).
Conclusions: pIL-12 EP monotherapy induces objective tumor responses in a significant proportion of patients (BORR 31%) and treatment was well tolerated. pIL-12 EP promotes the expression of pro-inflammatory genes including genes required for antigen processing and presentation. Regression of non-treated lesions suggests successful induction of systemic anti-tumor immune-mediated effects. Based on these data, further investigation of pIL-12 EP both as a single agent, and in combination with other therapies such as anti-PD1/PD-L1, is warranted.

POSTER PRESENTATIONS

MOLECULAR AND IMMUNO ADVANCES

P1

AurkA inhibitors enhance the effects of B-RAF and MEK inhibitors in melanoma treatment

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Journal of Translational Medicine 2015, **13(Suppl 1):**P1

Background: Aurora Kinase A (AurkA), one of the key regulators of M phase progression, is over-expressed in melanoma and has been observed to limit tumor growth [1,2]. The potential use of this molecule as target for biological therapy in melanoma has been examined.
Materials and methods: A375mel (BRAFV600E) melanoma cell line was used in this study. The cell line was exposed to B-RAF inhibitor (GSK2118436), MEK inhibitor (GSK1120212) and AurkA inhibitor (MLN8054) as single agents or in various combinations (B-RAF plus AurkA inhibitor, MEK plus AurkA inhibitor) or in triple combination (B-RAF plus MEK plus AurkA inhibitor).
The effects on the cell growth of drugs, used as single agents and as different combinations, were examined by the xCELLigence technology. Total protein extracts were examined for p53 and c-myc protein expression by Western Blot analysis. The drug's efficacy was also tested by using a 3D-human melanoma skin reconstruction model.
Results: A375 (BRAFV600E) melanoma cells treatment with AurkA inhibitors in combination with B-RAF and/or MEK inhibitors alone and/or with both B-RAF/MEK inhibitors, increased the anti-tumor efficacy of the drugs than given as single agents.
The AurkA inhibitors enhancing anti-melanoma effect on B-RAF and MEK inhibitors was furthermore confirmed in a 3D-human melanoma model, where it was restricted to a melanoma cell sub-population localized at epithelial/dermal junction site. However, S-100 and Ki-67 positively stained spindle-shaped cells were detected in the dermal stratum, suggesting the presence of alive and proliferating melanoma cells.
Conclusions: These findings provide new prospects for melanoma research. For the first time, based on these results, it was observed that the triple combination treatment was more efficacious as anti-melanoma therapy. Interesting, the treatment was efficacious only on polygonal-shaped melanoma cells present at the epidermal/dermal junction site as small nests, while spindle-shaped melanoma cells present in the dermal stratum remained alive and proliferating. This finding suggested that these cells may account of the drug resistance and so be responsible of disease recurrence later on. Molecular characterization of these dermal cells may be critical for the development of novel therapeutic strategies.

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P2

Exome sequencing in primary melanoma identifies novel drivers of melanoma progression

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Background: Melanoma is the most aggressive skin cancer due to its high metastatic propensity and resistance to most traditional chemotherapeutic drugs [1,2]. At early stage melanoma can be cured by surgical excision, whereas metastatic melanoma is a highly lethal condition. To understand melanoma progression is crucial identify mutations that are involved in making an individual melanoma competent for metastatic spread. The most frequent known oncogenic mutation in melanoma is *BRAF*-V600E and several whole exome sequencing studies have revealed numerous other alterations [3-6]. It is well established that the aggressive behavior of melanoma is highly correlated with histological features, such as the thickness of the primary tumor and the mitotic index. Here we performed whole exome sequencing of 5 thin (<1mm in thickness) and 5 thick (>4mm in thickness) primary melanomas compared to matched-normal DNA.
Materials and methods: We have collected 10 fresh primary melanomas from 10 untreated patients: DNA samples from melanoma tissues and peripheral blood (normal DNA) were available from all of the recruited patients. Genomic DNA was extracted from tumor and peripheral blood samples using the QIAamp DNA Minikit, (Qiagen, Hilden, Germany). Extracted DNA was used for Next-Generation Sequencing analysis by Illumina.
Results: We confirmed recurrent somatic mutations in known melanoma-related genes, including BRAF, c-KIT, EGFR, PPP6C, MLL3 and several components of the glutamate signaling. In addition, we discovered mutations in genes not previously linked to this tumor, such as CSMD1, FGFR4 and components of the Hedgehog (HH) signaling pathway. In particular, in a thick melanoma we found a novel activating mutation in the transcription factor GLI1, one of the final effectors of the HH signaling. Additionally, we identified candidate metastasis-driving mutations such as ADAMTS6, ADAMTS7, CHD9, MLL3, NALCN and TSC2 in the 3 thick melanomas that produced metastasis. Interestingly, we identified several regions of focal somatic copy-number alterations (SCNAs) that were altered at significantly higher frequency in thick compared to thin melanomas. Several gene families are comprised among these regions of focal SCNAs, including components of Notch, HH and Wnt/β-catenin signaling pathways, BRAF, c-MYC and its cofactor PIM1, several ADAMs, EGFR and the HOX genes.
Conclusion: Our data identify potential drivers of melanoma progression, enhancing our understanding of the genomic complexity underlying melanoma.

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P3

The relevance of BRAF G469A mutation in determining the response to therapy in metastatic melanoma

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Background: BRAF G469A is a missense mutation within exon 11 of the BRAF gene resulting in a constitutively active state of the enzyme responsible also for the protein localization to the mitochondria. The BRAF G469A mutation, frequently recovers in lung cancer, is rare in melanoma and uncertain is its association with a more aggressive disease. BRAF G469A mutation is frequently associated to MAP kinase cascade signaling activation, however no evidence currently exists about its role in determining sensitivity/resistance to BRAF inhibitors vemurafenib or dabrafenib. In our Institute, a patient with metastatic melanoma (MM) was treated with fotemustine, however the disease progressed. From patient biopsy, a new metastatic melanoma cell line has been established and named Mo-1. Here, we investigated the sensitivity of Mo-1 cells to vemurafenib and abraxane, both already approved for the treatment of melanoma carrying BRAF V600 mutations, comparing it with that found in MM cells wild type for BRAF or mutated in V600. Furthermore, a biomarker for the response to abraxane is hypothesized.
Materials and methods: We tested the sensitivity of Mo-1 (BRAF G469A), HBL and LND-1 (BRAF wild type), MBA72 and Hmel-1 (BRAF V600) to abraxane and vemurafenib by MTT cytotoxicity assay. In addition, cellular effectors were investigated by ELISA kits, western blotting and flow cytometry.
Results: The exposure to vemurafenib leads to a progressive inhibition of proliferation however, the concentrations utilized are high and similar to those utilized in MM models constitutively resistant to BRAF inhibitors. Thus, the BRAF mutation G469A confers resistance to vemurafenib also confirmed by the partial response obtained when the patients was treated with the drug. The cellular effector responsible for the resistance might be the activation of Erk1/2.
The binding of BRAF G469A to the outer mitochondrial membrane suggested to evaluate the mitochondria functionality in Mo-1 cells. An high production of ATP and the release of lactate were evident.
Finally, abraxane, which induces mitochondria-mediated apoptosis, strongly reduced proliferation in Mo-1 cells. Possible biomarker responsible for this response is the very low expression level of PMEL17, transcriptionally regulated by MITF, which is known to be negatively involved in determining the activity of taxanes.
Conclusion: Thus, the mutation BRAF G469A in patients with MM should be related to a weak effectiveness of therapy with BRAF inhibitors and a promising therapeutic approach may be with abraxane.

P4

microRNAs and next generation sequencing for the prognosis of the metastatic melanoma

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Journal of Translational Medicine 2015, **13(Suppl 1):**P4

Background: Melanoma is the most serious form of skin cancer because of its increasing incidence. Recent studies demonstrated the involvement of specific microRNAs in the melanoma initiation, progression, diagnosis and prognosis. Currently the treatment for metastatic melanoma with the mutation of BRAF V600E expects the Vemurafenib that blocks mutated BRAF protein leading to cell-cycle arrest. Unfortunately, the reactivation of MAPK signalling or activation of an alternative signalling pathway, as PI3K/AKT/mTOR, deriving from different mechanisms of acquired tumor drug resistance (as secondary mutations in NRAS or MEK1) causes disease progression within 6–8 months after the therapy beginning. The aim of this work was to evaluate a possible MAPK reactivation due to microRNAs involvement or to unclassified gene variants not yet associated to metastatic melanoma therapy response.
Materials and methods: A set of 43 patients, treatment naïve and with confirmed histological stage IV of metastatic melanoma was enrolled through the Oncology Unit of the IRCCS "Giovanni Paolo II" in Bari, Italy. Thirty melanoma cases were BRAF mutated at the codon 600, while 13 were wild type. We analyzed 15 microRNAs and the correspondent target genes by TaqMan probes. Moreover we developed an Ampliseq Custom panel for ION Torrent PGM Sequencer to analyze the coding region of several target genes with a coverage of 93.85%. The correlation between microRNA expression signature and the detected mutations with time to progression of patients treated with Vemurafenib has been analyzed.
Results: High expression of miR-192 and miR-193b* and low expression of miR-132 resulted associated with short time to progression, by the Kaplan-Meier survival curves, indicating a poor prognosis. MiR-193b*was also included in the results of the multivariate Cox analysis that revealed a significant signature of 11 microRNAs (miR-34a, miR-146b, miR-182, miR-155, miR-101, miR-222, miR-21, miR-338-3p, miR-193b*, miR-193a and miR-191) whose high expression was associated to worse prognosis. The univariate Cox model confirms that potential role for miR-193b* as poor outcome marker. Moreover we detected mutations associated with the response to different therapeutic approach and, more interestingly, we identified gene alterations not yet associated to this pathology.
Conclusions: Our study highlighted the prognostic role of microRNAs in metastatic melanoma. Furthermore the use of Next Generation Sequencing to detect known or novel mutations could be useful in clinical practice.

P5

Melanoma cells with acquired resistance to dabrafenib display changes in miRNA expression pattern and respond to this drug with an increase of invasiveness, which is abrogated by inhibition of NF-κB or the PI3K/mTOR signalling pathway

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Journal of Translational Medicine 2015, **13(Suppl 1):**P5

Background: The therapeutic success of BRAF inhibitors (BRAFi) is limited by the emergence of drug resistance [1,2]. Although several mechanisms underlying acquired resistance to BRAFi have been identified [1,2], further studies are required to disclose their entire spectrum. Altered expression and/or function of microRNAs (miRNAs) is involved in tumor onset, progression and drug resistance [3]. Here, we determined miRNA expression profiles of melanoma cells sensitive or resistant to the BRAFi dabrafenib, and investigated the effect of this drug on their invasiveness. We also evaluated the consequences of inhibiting NF-κB or the PI3K/mTOR signalling pathway on the invasive capacity of dabrafenib-resistant cells.
Materials and methods: The $BRAF^{V600E}$ mutant A375 melanoma cell line, and its dabrafenib-resistant subline A375R, generated in our laboratory, were analyzed for the levels of total and phosphorylated ERK1/2 and AKT, by Western blotting, and for miRNA expression pattern by using Affymetrix miRNA 3.1 arrays. A375 and A375R cells were also exposed to dabrafenib (100 nM, 48 hours) and tested for: a) in vitro invasion of extracellular matrix (ECM), under basal conditions and in response to VEGF-A (Matrigel covered Boyden chambers); b) VEGF-A secretion (ELISA). Finally, in vitro ECM invasion by A375R cells treated with the NF-κB inhibitor NEMO-binding domain (NBD) peptide (50 μM, 48 hours) or the PI3K/mTOR inhibitor GSK-2126458 (20 nM, 48 hours), alone or in combination with 100 nM dabrafenib, was evaluated.
Results: A375R cells showed higher levels of phospho-ERK and phospho-AKT as compared with A375 cells. Eighty-nine miRNAs were up-regulated and 47 miRNAs were down-regulated in the A375R cells with respect to A375 cells. Gene Ontology analysis of the putative target genes of the top-ten down-modulated miRNAs revealed "regulation of cell motion" and "regulation of cell migration" as being among the most significantly enriched terms. A375 and A375R cells differently responded to dabrafenib, which strongly inhibited invasiveness and VEGF-A secretion in A375 cells, whereas it stimulated these functions in A375R cells. Treatment of A375R cells with NBD-peptide or with GSK-2126458 inhibited spontaneous invasion of ECM. Moreover, both agents completely abrogated dabrafenib-induced stimulation of ECM invasion.
Conclusions: Our data show that changes in miRNA expression occur in melanoma cells with acquired resistance to dabrafenib, and that invasiveness of these cells is enhanced in the presence of the drug. They also indicate that targeting NF-κB or the PI3K/mTOR pathway could be an efficient therapeutic strategy in patients who develop resistance to BRAFi.
Acknowledgment: Supported by the Italian Ministry of Health, grant 5PerMille-2010
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P6

Neuropilin-1 expressing melanoma cells as a model to study the aggressiveness of metastatic melanoma

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Background: The molecular mechanisms associated with the acquisition of a metastatic phenotype by melanoma cells are not very well understood. Therefore, the identification of molecular determinants involved in the metastatic switch that may either cause or contribute to the aggressiveness of melanoma is of primary relevance.
We had previously identified neuropilin-1 (NRP-1), a co-receptor of the vascular endothelial growth factor-A (VEGF-A), as an important determinant of melanoma aggressiveness, in clones of the human melanoma cell line M14, expressing or not NRP-1 [1,2]. We demonstrated

that even though the simultaneous presence of both VEGFR-2 and NRP-1 potentiates VEGF-A secretion and the aggressiveness of melanoma cells, NRP-1 is by itself able to promote cell invasion [1].
During melanoma progression, tumour cells show increased adhesiveness to the vascular wall, invade the extracellular matrix (ECM) and frequently form functional channels similar to vascular vessels (vasculogenic mimicry) [3]. In the present study we analysed the mechanisms responsible for the aggressive phenotype of NRP-1 expressing melanoma cells.
Materials and methods: Melanoma aggressiveness was evaluated *in vitro* as cell ability to migrate through an ECM layer in Boyden chambers and to form tubule-like structures on matrigel gels. Pre-incubation of the cells with specific blocking antibodies allowed the identification of specific integrins and other molecules relevant to these processes. The results obtained by anti-integrin antibodies, showing the involvement of $\alpha v\beta 5$ integrin in the aggressiveness of melanoma cells expressing NRP-1, were confirmed by *ITGB5* gene silencing and by the use of cilengitide, a potent inhibitor of αv integrins activation.
Results: The expression of $\alpha v\beta 5$ integrin was found to be twice higher in NRP-1 expressing melanoma cells than in the low-invasive NRP-1 negative control. Its blockage resulted in a significant decrease of the ability of NRP-1 expressing cells to invade ECM and to form tubule-like structures on matrigel. Cilengitide and *ITGB5* silencing reduced ECM invasion and vasculogenic mimicry. Moreover, cilengitide down-modulated the secretion of VEGF-A and metalloproteinase-9 (MMP-9). Finally, melanoma cells expressing NRP1, but lacking other VEGF-A or PlGF receptors (VEGFR-1 and VEGFR-2), specifically responded to PlGF in a chemotactic assay.
Conclusions: In conclusion, we identified novel mechanisms that modulate melanoma aggressiveness involving NRP-1, $\alpha v\beta 5$ integrin and PlGF, which might be considered as new targets of therapeutic strategies to inhibit the metastatic disease.
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COMBINATION THERAPY

P7

Safety and efficacy of vemurafenib in BRAF V600E mutation-positive metastatic melanomas

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Background: Metastatic melanoma has a poor prognosis, and 5-year survival is 65% with regional stage disease and 15% with distant stage disease. Chemotherapy has limited success in metastatic melanoma, with a median overall survival of 8 months. Malignant melanoma is not a singular, homogeneous disease but rather a mixture of subtypes characterized by specific mutations. Tumours with BRAF mutations respond to BRAF kinase inhibitor vemurafenib, that was approved by US FDA in 2011 and EMA in 2012 for therapy of patients with advanced melanoma, harboring mutation in BRAF V600E gene. Some randomized clinical trials focused on the significant reduction in the risk of death and disease progression associated with vemurafenib, compared with classical standard chemotherapy. Vemurafenib is generally well tolerated with the most common side effects being arthralgia, photosensitivity, fatigue and dermatitis.
Materials and methods: From January 2013 to August 2014 we have collected data of 16 patients with metastatic melanoma (IV stage). Metastatic sides were: brain (35%), liver (29%), skin (24%) and lung (22%). We estimated overall survival (OS) and toxicities therapy-related. Patients were eligible if their tumour tissue was positive for the presence of BRAF V600E mutation. Dosing of vemurafenib ranged from 480 mg/bid to 960 mg/bid. Each patient has been revalued every two weeks with clinical examination until conclusion of treatment. Treatment was discontinued on disease progression or toxicity.
Results: Median overall survival was 13 months. The most common adverse effects included arthralgia (40%), fatigue (35%) and photosensitivity reactions (25%), grade 1 or 2 side effects as per the Common Terminology Criteria for Adverse Events (CTCAE). Elevated liver enzymes were documented in close to 10% of treated patients and no prolongation of the QTc interval, cardiac arrhythmias, keratoacanthoma and squamous cell carcinoma were reported. Dosing's modification has been required in three patients: two patients have discontinued the treatment and they have been resumed at 720 mg/bid while one patient have been resumed at 480 mg/bid. Nobody needed to stop the treatment due to unacceptable toxicities.
Conclusions: According to the findings available in literature, inhibition of BRAF improves clinical outcome in patients with the BRAF V600E mutation. Vemurafenib was well tolerated and adverse event profiles were similar to those reported in literature.

P8

BRAFV600E mutation positive metastatic melanoma in a young woman treated with anti-BRAF/anti MEK combination: a case report

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Background: The recent Combi-v [1] and Combi-d [2,3], phase III randomized trials, showed, respectively, an OS benefit with Dabrafenib/Trametinib combination versus Vemurafenib and an improvement in PFS and ORR with the same combination versus Dabrafenib alone, in BRAF V600E/K mutation positive metastatic or unresectable cutaneous melanoma. We report the case of a young patient with metastatic melanoma treated with antiBRAF/antiMEK combination.
Case report: A 36-years woman referred to our institution in March 2014, after the diagnosis of metastatic melanoma. ECOG Performance Status was 1. No comorbidities, no previous melanoma treatment. In May 2011 the patient reported a traumatic event which resulted in the removal of a mole localized in the right thigh. Since August 2013 she noted a swelling in the right area of the groin. It increased constantly causing pain, deambulation problems and limiting daily activities. On January 2014, due to the worsening of the swelling and pain, which resulted in the patient becoming unable to maintain a sitting position, she underwent an ultrasound exam that displayed pathological lymph nodes. A FNAB showed a metastatic localization of melanoma, S 100+++, Hbm 45++. BRAFV600E mutation was detected with Cobas 4800 BRAF mutation test. A CT/PET displayed conglobated pathological lymph nodes in right groin of around 10 cm; right iliac obturator lymph node of 6 cm and osteolytic lesion at sacroiliac articulation. In April 2014, after adequate screening, the patient began Dabrafenib/Trametinib combination under the Compassionate Use program. Just after one month of treatment she reported clinical benefit in terms of deambulation improvement and pain relief. During the second month of therapy the treatment with Dabrafenib was withdrawn for some days, due to two episodes of hyperpyrexia up to 39°C, treated with Paracetamol. Progressively the antiBRAF drug was reintroduced with no further interruption. The CT/PET performed after three and six month of treatment showed a good response to the therapy with a dimensional decrease and SUV reduction of more than 50% in all target lesions. Currently the patient is continuing the treatment; her ECOG PS is 0. She does not require any antalgic drugs. Our aim is obtain the maximum reduction to allow a surgical approach.
Conclusion: In our case the Dabrafenib/Trametinib (antiBRAF/antiMEK) combination leads to a fast clinical and radiological disease response with a good toxicity profile and a good management of AEs.

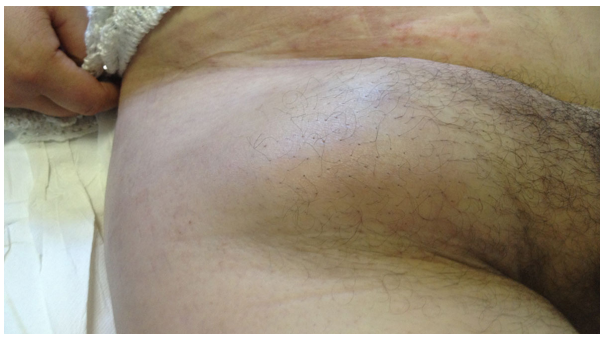

Figure 1(abstract P8) March 2014

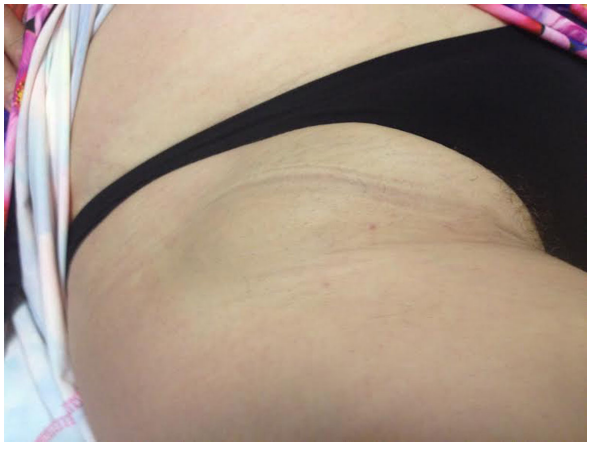

Figure 2(abstract P8) September 2014

Written informed consent was obtained from the patient for publication of this abstract. A copy of the written consent is available for review by the Editor of this journal.

Acknowledgment: GSK authorized this abstract

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TUMOR MICROENVIRONMENT AND BIOMARKERS

P9

Simultaneous determination of two serum tumor markers in assessing malignant melanoma patients

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Journal of Translational Medicine 2015, **13(Suppl 1):**P9

Background: The incidence of cutaneous malignant melanoma (MM) continues to raise despite extensive prevention programs. If localized disease can be cured, the prognosis of metastatic melanoma is grim. Therefore, reliable methods to detect patients at risk for disease progression are sought. Field of tumor markers has captured attention because it allows the first steps toward personalized medicine. A positive tumor marker has a limited ability to correctly detect all sick people, so it was proposed to simultaneously measure several markers in order to improve their performance [1,2]. S100 and Melanoma Inhibitory Activity (MIA) are most frequently used for monitoring MM patients. Both proteins have a high specificity and their expression correlates with body tumor burden [3].
Materials and methods: Between 2009 and 2013, we determined MIA and S100 serum concentration in 120 patients with non-metastatic MM and 50 healthy donors, in order to compare diagnostic and prognostic potential of these two biomarkers. Both proteins were measured by a high sensitivity ELISA method. For S100, a threshold of 100 ng/L was accepted, as recommended by the kit manufacturer. Using the ROC curve, we estimated a MIA cut-off level of 9.4 ng/mL [4]. Patients were divided into 4 groups according to markers concentrations: both markers positive, both negative and one positive/one negative. Median, disease free and overall survival (OS) were estimated for each group.
Results: Survival varied depending on the number and type of markers exceeding the cut-off. Median survival decreased in this order: from S100/MIA negative group to MIA negative/S100 positive, MIA positive/S100 negative, S100/MIA positive group. It seems that a MIA value above the cut-off has a negative impact on OS greater than an increased S100 value. Two years OS was significantly higher in MIA/S100 negative group compared with MIA/S100 positive one (81%/51%; p=0.05). Furthermore, patients with a single positive marker had a higher OS than those with both markers increased.
Conclusions: Simultaneous use of S100 and MIA increased sensitivity of identifying MM patients irrespective of clinical stage. Several tumor biomarkers determination affords selection of those produced in high volume that will be further used in patients follow-up. Measuring both MIA and S100 allows outlining of an intermediate prognosis group of patients, represented by those with a single positive marker, who have a lower risk of relapse and death than those with both positive markers.
Acknowledgment: This paper was co-financed from the European Social Fund, through the Sectorial Operational Programme Human Resources Development 2007-2013, project number POSDRU/159/1.5/S/138907 "Excellence in scientific interdisciplinary research, doctoral and postdoctoral, in the economic, social and medical fields -EXCELIS", coordinator The Bucharest University of Economic Studies.

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P10

Clinical value of Melanoma Inhibitory Activity in stratifying malignant melanoma patients

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Journal of Translational Medicine 2015, **13(Suppl 1):**P10

Background: Malignant melanoma (MM) is a heterogeneous disease, well-known for its unpredictable clinical course and lack of response to most systemic therapies. Furthermore in some parts of the world it has quite an epidemic nature, with an incidence that has increased steadily in the last 30 years [1]. Therefore the characterization of new tumor markers that could better suggest the patient outcome has become a priority for many research centers. Melanoma Inhibitory Activity (MIA) is a protein highly expressed and secreted by malignant melanocytes [1,2], without being identified in benign melanocytes or other normal skin cells [3]. More, the induction of MIA synthesis seems to be an early event in carcinogenesis, taking into account that all in situ tumors express it. The objective of this study was to assess the significance of increased serum MIA concentration in MM patients with no metastases at primary diagnoses.
Materials and methods: Between 2008-2012 we have collected 200 blood samples from 150 patients with non-metastatic MM and 50 healthy donors. The patients were staged according to TNM classification 2009 as follows: 28 in stage I, 72 in stage II and 50 in stage III. The blood was withdrawn after the primary tumor excision and before any other treatment. MIA was measured by a high sensitivity ELISA method, with a kit produced by Roche Diagnostics. Using the ROC curve, MIA cut-off value was set at 9.4 ng/ml [4].
Results We estimated overall survival (OS) and disease free survival (DFS) for the entire lot and for each stage separately, according to MIA cut-off level. The length of follow-up was 44 month, from the moment of MIA measurement. Univariate Cox regression suggested that patients with an increased MIA serum concentration had a three times higher risk of relapse (HR=3.3895) and death (HR = 2.7597) than patients with values under the calculated threshold (p=0.000). More important is that MIA keeps statistical significance in multivariate analyses, predicting risk of death or relapse after corrections for clinical stage.
Conclusions: In our study, MM patients with a MIA serum concentration above 9.4 ng/ml had a worse DFS (p=0.0109) and OS (p=0.0009) than patients with values below 9.4 ng/ml. We consider that MIA serum concentration is a valuable prognostic factor and could become a tool for selecting patients at risk for developing metastases rendering them eligible for neoadjuvant treatment.
Acknowledgment: This paper was co-financed from the European Social Fund, through the Sectorial Operational Programme Human Resources Development 2007-2013, project number POSDRU/159/1.5/S/138907 "Excellence in scientific interdisciplinary research, doctoral and postdoctoral, in the economic, social and medical fields -EXCELIS", coordinator The Bucharest University of Economic Studies.

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P11

Melanoma Inhibitory Activity and regional lymph node status in malignant melanoma patients

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Journal of Translational Medicine 2015, **13(Suppl 1):**P11

Background: For many cancers, and malignant melanoma (MM) makes no exception, regional lymph node (RLN) status is decisive for subsequent patients' outcome. But particularly for MM, stage III comprises a very heterogeneous group of patients, from those with microscopic invasion diagnosed by sentinel node (SN) biopsy to those with matted nodes. Correct staging of patients with clinically free RLN requires SN biopsy. Because this is an invasive maneuver, various substitutes were searched. Thus, in literature there are conflicting references to a possible link between Melanoma Inhibitory Activity (MIA) serum concentration and SN status [1,2]. MIA, a protein secreted by malignant melanocytes into the extracellular space, blocks the melanoma cells attachment to fibronectin and laminin. Thus MIA increases malignant cells mobility and promotes local invasion and metastasis [3]. In this context, we sought a connection between RLN status and MIA serum concentration in our group of patients.
Materials and methods: 150 patients with non-metastatic cutaneous MM were treated in our clinic between 2009 and 2013. They were staged according to 2009 AJCC (American Joint Cancer Committee) staging system. 47 patients were assigned to stage III: 37 presented with clinically evident lymphadenopathies and 10 had positive SN. SN biopsy was performed in 61 patients with intermediate thickness MM and no clinical or ultrasound signs of RLN metastasis. MIA was measured preoperatively in all patients. A cut-off value of 9.4 ng/mL was calculated using ROC curve [4]. Patients were divided in four groups according to RLN status (N0/N1/N2/N3).
Results: Mean and median MIA serum concentration progressively increased along with the number of metastatic RLN. The difference between mean and median MIA values in the 4 groups was not accidental, but the consequence of different tumor load (p = 0.001). Only N0 patients had mean and median MIA concentrations less than 9.4 ng/mL, for all the other N categories, values surpassing the threshold. SN identification rate was 100%. The mean and median MIA serum value in SN positive group were higher than in SN negative one, but the differences were not statistically significant (p=0.6191) and in both cases didn`t exceed the upper normal limit.
Conclusion: MIA serum concentration increases with RLN tumor burden. In our study SN status didn`t correlate with MIA value, but the small number of patients prevents us to draw a conclusion.
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P12

Immune infiltrates impact on the prediction of prognosis and response to immunotherapy of melanoma patients

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Background: Melanoma is a highly malignant melanocyte-derived tumor and its incidence is increasing at outstanding rate. Despite specific therapies have been explored for many years, no effective therapeutic options have been developed. Vaccination strategies, including Dendritic Cells (DC) based immunotherapy, are consistently increasing the proportion of cancer patients with anti-vaccine immune responses although the number of patients with increased overall survival is still limited. The efficacy of the immunotherapy is mainly dependent on tumor microenvironment –immune system interactions.
Our aim is to evaluate the host immune response to melanoma by quantifying the density and location of T cells in primary tumors of patients treated with DC immunotherapy and correlate them with clinical variables such as overall survival (OS).
Materials and methods: We collected 60 FFPE primary tumors from melanoma patients treated with DC immunotherapy. Serial sections (4 micron in thickness) were stained with CD3, CD8 and CD45RO antibodies. Haematoxylin was used as a counterstain and Nova Red for the immunohistochemical stain. All the slides were digitalized and an automated quantitative analysis was performed in order to evaluate the density and location of two lymphocyte populations, cytotoxic (CD8) and memory (CD45RO) T cells. Of all samples the clinical outcome of the patient is known.
Results: The immunohistochemical analysis of primary melanoma using a small set of patients resulted in significant differences between short (OS<12 months) and long survivors (OS>24 months). A high degree of T cells infiltration was seen in the tumor area of both patients, responding and non-responding to DC immunotherapy. However, the location but not the density of TILs was significantly different in the two cohorts of patients and showed a strong correlation with clinical response to DC vaccination.
Conclusions: Immune cells within melanoma tumors may have a prognostic value and clinical significance as a predictor of patient outcome and response to DC immunotherapy in melanoma patients.

P13

Gender differences and outcome of melanoma patients

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Background: In the United States, 80,000 cases of melanoma are expected in 2014, representing 5% and 4% of all diagnosis of cancer in men and women, respectively.
In Italy in 2013, there were an estimated 10,500 cases of cutaneous melanoma, with an incidence of 14.3 cases per 100,000 male population and 13.6 cases per 100,000 female population.
Female melanoma patients generally exhibit significantly longer survival than male patients.
The aim of this study is to evaluate the role of gender in survival of melanoma patients and the relationship between gender and pathologic features of the neoplasm.
Materials and methods: In this retrospective study, we examined 1,023 consecutive patients treated at the Department of Medical Oncology and at the Department INRCA-IRCCS of Dermatology in Ancona, Italy from February 1987 to March 2014. Survival analysis was conducted via Kaplan-Meier product-limit method and the Mantel-Haenszel log-rank test was employed to compare survival among groups. A significance level was set at a 0.05 value and all *p* values were two-sided.
Results: Of the total of 1,023 patients examined, 47.6% were men and 52.4% were women.
Pathological features discerned by gender were the following. Thin Breslow thickness (pT1) was more common in women versus men (45% vs 38.6%); Breslow thickness >1 mm (pT2) represented 16% of melanomas both in women and men; but thick melanomas (pT3-pT4) resulted more frequent in men. The presence of histologic ulceration in melanoma was approximately 80% and 9.7% in men and women melanoma respectively. Finally "non brisk" TILs pattern was founded comparable in men and women (25%), rather absent TILs pattern proved as a result more characteristic of men melanoma.
Subgroups analysis showed that women had a significant advantage in 12-year disease free survival (DFS) and 12-year overall survival (OS) adjusted for Breslow thickness (T1-T2 melanomas, p<0.001), presence of histologic ulceration (p<0.001), absent regression (p<0.001), "non brisk" TILs pattern (DFS and OS, respectively p=0.005 and p=0.009) and "absent" TILs melanomas (p<0.001).
Globally, a significant female advantage was observed for overall survival (p<0.001).
Conclusions: Our results show that women have a consistent and independent survival advantage compared with men after adjusting for many variables indicating that factors other than pathologic features reduce mortality risk in female melanoma patients. We aim to further investigation about possible biologic sex differences in tumor-host interactions.

P14

Circulating dendritic cell levels identify high-risk stage II-III melanoma patients: a potential role as additional prognostic marker

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Background: Melanoma is an immunogenic cancer that overcomes the immune surveillance through the production of tolerogenic cytokines and growth factors within microenvironment. Melanoma-derived dendritic cells (DCs) show altered maturation, cross-priming and antigenic presentation and their major defect concerns the activation of the STAT pathway. The prognostic criteria to define melanoma at high-risk of relapse/recurrence include the Breslow depth, the Clark level and number of mitosis. Sentinel lymph node (SLN) characterization is a prognostic factor in melanoma, although false negative occurs in approximately 5% of patients. We investigated the potential prognostic role of DC number variation in relation to clinical stage, and suggest their role as early predictor of high risk melanomas.
Methods: 84 patients (group A; stage I-IV) with a previously definite diagnosis of melanoma as well as 18 (group B) with highly suspected cutaneous lesion were enrolled into the study. Peripheral blood was collected at study entry in patients of group A, whereas cells from group B were collected before and after the primary tumor exeresis and then before/after any surgical procedure including SLN, lymphadenectomy or metastasis removal. Both myeloid (m) and plasmacytoid (p) DCs were investigated by flow-cytometry using the anti-Lin, -CD11c, -BDCA-1, -CD123, and -BDCA-2 MoAbs. The percentage number of both mDCs and pDCs was correlated to clinical stage as well as to indipendent prognostic factors as histological features including Breslow, Clark level, presence/absence of tumor-infiltrating lymphocytes, and BRAF $V600^{E/K}$ mutations.
Results: The percentage of both mDCs and pDCs from group A were similar in stage I-III and dramatically reduced in those at stage IV. A. Lack of correlation was also demonstrated with clinical features and prognostic factors. By contrast, data from group B showed: an increase of mDCs and pDCs (p<0.05) after tumor removal as well as in those with negative SLN whereas both subsets resulted unchanged in patients with positive SLN. A weak trend to DC increase occurred in patients undergoing negative lymphoadenectomy. Number of mDCs and pDCs

from group B was correlated with histological features and unrelated to mutational status.
Conclusions: These findings suggest the critical pathogenetic role of DC in melanoma and their measurement may be thus proposed as additional prognostic factor to limit the risk of underscoring the melanoma stage of patients at high risk of relapse/recurrence.

P15

Dendritic cell-derived exosomes (Dex) are potential biomarkers of response to Ipilimumab in metastatic melanoma

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Journal of Translational Medicine 2015, **13(Suppl 1):**P15

Background: Chemotherapy and vaccination with tumor-loaded dendritic cells (DCs) show poor impact on overall survival (OS) in metastatic melanoma[1,2]. Ipilimumab (IPI) improves OS throughout the blockade of CTLA4-mediated inhibitory signals in T-cells and restores the efficiency of the antigenic cross-priming by mature (m) DCs[3]. However, variation of immune cells to evaluate the response to IPI does not reflect the T-cell activation and is a modest predictor of clinical response. Recent studies in human and experimental melanoma demonstrated that mDCs release endomysial microvescicles namely dexosomes (Dex) showing a functional anti-melanoma activity as well as an antigenic profile resembling that of circulating mDCs including CD40, CD80 and CD86 co-stimulatory molecules.
This research is aimed to identify an early biomarker of T-cell activation for predicting the clinical response in IPI-treated melanoma patients.
Methods: Thirthy-four patients with metastatic melanoma were treated with IPI and sera collected at each infusion. Serum Dex were purified by the 'Total Exosome Isolation kit' (Invitrogen) and conjugated with magnetic beads of 4 μm of diameter (Dynabeads). Dex were first identified by size and then CD40, CD80 and CD86 expression was evaluated by flow-cytometry using relative MoAbs. The response to IPI was analyzed up to 12 weeks after the end of treatment, according to RECIST criteria. Moreover, DEX levels were compared with clinical and immunological parameters by the Mann-Withnet test.
Results: Both CD40 and CD80 expression was unchanged after the end of IPI treatment with respect to basal levels, whereas a significant increase of Dex-CD86 expression occurred as compared to baseline (21.3±1.5% vs 12.0±0.8%, $p<0.05$) in 5 patients with partial response and 1 in complete remission (19.4±1.2% vs. 9.9±1.1%). A weak trend to the increment of Dex-CD86 occurred in patients with stable disease, while those in clinical progression showed low levels in all instances. CD86 expression was apparently unrelated to LDH levels and absolute leukocyte count.
Conclusions: Level of CD86 expressed by Dex reflects the immunological activation in melanoma patients treated with IPI. Therefore, the measurement of soluble Dex-CD86 could be an early marker of response to IPI and predict the efficiency of immunological response.

P16

"Immune B Cells know it better": tumorimmunological panel assay to define tumor-associated antigen binding antibodies in patients with metastatic melanomas

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Background: Revealing novel cancer targeting biomarkers is a great challenge, and especially urging in cancer types with a more pronounced metastatic feature. We focus on potential anti-tumor immune reactions of the host. In order to harness the natural humoral immune response a novel immunological and molecular genetic panel assay has been developed for the investigation of patients with melanomas.
Materials and methods: Punch biopsies were taken of surgically removed fresh cancerous tissues and peripheral blood was gathered from the patients involved into the study (n= 125). Ethical permission was provided by the Scientific and Research Ethics Committee of the Medical Research Council of the Hungarian Ministry of Health (ETT TUKEB 16462- 02/2010). We established and standardized two experimental strategies (Epstein Barr virus transformation and cloning with limiting dilution assay (LDA) and tumor infiltrating B cell (TIL-B) antibody phage display technology) and started basic processes for a detailed immunglobulin repertoire analysis at DNA level. We set up a novel native tumor cell membrane preparation technique extremely useful for specific detection of tumor reacting antibodies or antibody fragments (eg: immunoblotting, scFv phagemid ELISA). Defining essential tumor-associated antigens on the cancerous tissue specimen by immunohistochemistry became the other part of the tumorimmunological panel assay. Results: We claim that this complex quantitative and qualitative analysis of antibodies in sera and in the tumor microenvironment results in revealing tumorspecific antibodies of human origin. Our antibody profile analysis revealed glycoprotein and sialylated glycolipid based tumor-associated antigen-specific antibody-variable regions in various patterns.
Conclusions: The present technological developments enable the specific detection of cancer associated sialylated glycolipid and glycoprotein antigens with unique characteristics. The study helps to understand the question of "abnormal glycosilation" and its role in cancer. We conclude that the complex tumorimmunological assay has important potentials in evaluating the host's anti tumor immune status.
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