Report from the II Melanoma Translational Meeting of the Spanish Melanoma Group (GEM)

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Abstract: The II Melanoma Translational Meeting of the Spanish Melanoma Group (GEM) was held in Barcelona, Spain, at the Hospital Universitari Quiron-Dexeus on November 3rd, 2016. The conference featured leaders in the fields of oncology, immunology and dermatology, all working at both national and international levels on basic research with direct applications to the clinical setting. The objective was to present cutting edge research on melanoma, mainly from Spanish research groups, but also from some international groups, with the aim of generating a network for future national and international collaborations. During a single day, fifteen speakers, seven biologists and eight clinicians, with a special focus on translational research, outlined the main findings of their ongoing studies. The moderators in every session were recognized GEM oncologists who guided discussion from the clinical point of view regarding the preclinical data presented. Herein, we summarize the main topics discussed during the meeting.

Keywords: Melanoma; meeting; Spanish; translational

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Introduction

The meeting was opened by Dr. Alfonso Berrocal, GEM Vice President, and by Dr. Gonzalez-Cao, Scientific Secretary.

The meeting was divided into six parts. The first was entitled "Inspired talks" and featured results of ongoing research in cancer immunotherapy from renowned researchers in the field, including Dr. Gajewski from Chicago University, Dr. Rosell from Hospital Universitari Quiron-Dexeus, Barcelona, and Dr. Wellbrock from Manchester University.

The second part was dedicated to reflections on research and society, during which Rosina Malagrida, from the

Living Lab for Health, at the Institute for AIDS Research IrsiCaixa, Barcelona, discussed the importance of generating research with practical applications to better respond to the needs of society. She talked about the new tendency to create multi-stakeholder platforms devoted to change the way Research and Innovation are performed. This new approach to R&I is promoted by the European Commission and also by many different stakeholders in Europe, under the name of Open Science and Open Innovation initiatives, in line with the so called "Responsible Research and Innovation (RRI)". Under this new paradigm, Research and Innovation governance is more open and inclusive, calling for the participation of interested members of society and involving methodologies that tend towards co-creation and the bringing together of patients, careers and clinicians, to collaborate during different phases of the process, such as the identification and prioritization of research topics about the effects of treatments.

The third part of the meeting touched upon novel techniques for the analysis of cell free circulating tumor DNA (cfDNA). Whole exome sequencing (WES) was discussed by Dr. De Matos-Arruda, a visiting clinician scientist from Cancer Research UK-(Cambridge) and Vall D'Hebron Institute of Oncology (Barcelona), multiple analysis platforms were discussed by Dr. Molina from Hospital Universitari Quiron-Dexeus, Barcelona, and RNA expression analysis was discussed by Dr. Karachaliou, from the same institution.

The fourth part of the meeting included talks from different scientific groups working on cancer research in Spain: Dr. Seoane, from Vall D´Hebron Research Institute, Barcelona, spoke about his research on the TGFB pathway; Dr. Puig, Head of the Dermatology Department at Hospital Clinic, Barcelona, spoke about melanoma susceptibility; Dr. Escors, from Navarra Biomed Institute, Pamplona, spoke about molecular mechanisms on the PD-1/PD-L1 pathway; Dr. Vaque from Hospital de Valdecilla, Santander, commented on the application of next-generation sequencing (NGS) analysis in skin cancer and, finally, Dr. Alvarez, from the same institution, explained her own results generating a dendritic cell (DC) vaccine for melanoma treatment.

In the final session, investigators of the Spanish Melanoma Group presented their ongoing projects. Dr. Gonzalez-Cao from Hospital Universitari Quiron-Dexeus, Barcelona, reviewed the role of the PD-1 pathway in chronic viral infections, such as HIV, and presented a clinical trial with an anti PD-L1 drug for cancer treatment in HIV infected patients. Dr. Prat, head of the Medical Oncology Service at The Hospital Clinic, Barcelona, presented the updated results of the study using NanoString Ncounter technology as a predictive tool for immunotherapy in cancer.

The meeting was closed by Dr. Paul Lorigan from Manchester University (UK) with an elegant talk reviewing the present and future of personalized medicine in melanoma.

Inspiring talks

In the first session, renowned researchers presented data from their recent work on predictive models and novel targets for immunotherapy. Currently, despite the high efficacy of anti-PD-1/PD-L1 and anti-CTLA-4 treatments, either as single agents or in combinations, the vast majority of patients with advanced melanoma will not respond to treatment and, finally, will not be cured.

Dr. Gajewski underlined several possible strategies for increasing the efficacy of these treatments. One option is the development of combinations with new drugs that inhibit other immunosuppressive molecules or activate pathways that attract lymphocytes into the tumor. Among the mentioned immunosuppressive molecules, he referred to indoleamine-2,3-dioxygenase (IDO). IFN-gamma from $CD8^+$ T cells upregulates the immunosuppressive molecules PD-L1 and IDO in the tumor, so inhibiting both could be synergistic as it has been demonstrated in mouse preclinical models (1). They conducted a phase I trial testing the anti IDO drug, epacadostat, in combination with the anti PD-1, pembrolizumab, demonstrating good tolerance and preliminary evidence of increased clinical activity (2). Regarding strategies to promote increased T cell infiltration, one opportunity could be the inhibition of β-catenin, previously described by Dr. Gajewski as a major mechanism explaining the presence or absence of tumor-infiltrating $CD8^+$ T lymphocytes (the so-called "inflamed or non-inflamed tumors") (3). Tumors that respond to immunotherapy require infiltration by $CD8^+$ T cells, and by a subtype of DC called Batf3 DCs which are critical for cross-presentation of class I MHC-restricted antigens to $CD8^+$ T cells (4). Tumor cell-intrinsic β-catenin prevents infiltration by these two immune cell lineages, arguing that β-catenin pathway inhibitors may have the potential to restore infiltration by these immunological cell types and

render such tumors immunotherapy responsive. Another approach that Dr. Gajewski's group is actively working on is the development of agonists of the STING pathway, an innate immune sensing pathway that can promote T cell activation and recruitment. Recent work has described the development of synthetic cyclic dinucleotides that activate murine and human STING alleles (5). When these agonists of STING are administered intratumorally, they achieve the activation of DC with the consequent priming of tumor-specific $CD8^+$ T cells, supporting rejection of treated tumors but also of tumors at distant sites (5). To conclude, Dr. Gajewski also commented on his recent data, published in Science in 2016, regarding the influence of intestinal microbiota in response to anti-PD-1/PD-L1 drugs in murine models, suggesting that a possible intervention on microbiota can amplify the anti-tumor immune response (6).

After Dr. Gajewski, Dr. Rosell, head of the Translational Cancer Research Unit from the Hospital Universitari Quiron-Dexeus in Barcelona, and a recognized leader in translational oncology, focused his talk on defining the main mechanisms of resistance to immunotherapy and the possible routes for therapeutic intervention. Alterations in the interferon-gamma pathway can provoke resistance to immunotherapy, since it is one of the well-known elements that leads to the acquisition of adaptive resistance to immunotherapy, promoting the expression of immunosuppressive factors, such as PD-L1, after recognition of antigen by T immune cells (7). Copy number alterations (CNAs) in genes of the interferon pathway, as well as amplifications of the inhibitory genes *SOC1* and *PIAS4*, are detected in more than 70% of melanomas resistant to immunotherapy (8). A recent publication in *Science* confirms that analysis of CNAs identified a higher copy number loss burden in non-responders to CTLA-4 and PD-1 blockade, and found that it was associated with decreased gene expression in immune-related pathways (9).

Secondly, activation of YAP1, already described by Dr. Rosell's group as a factor involved in resistance to targeted drugs (10,11), leads to the acquisition of an immuno-resistant phenotype by promoting the expression of immunosuppressive molecules EOMES and PD-1 (12), as well as the secretion of the chemokine CXCL5, leading to increased tumor infiltration by immunosuppressive cells, called myeloid derived suppressor cells (MDSCs) (13).

Also, the activation of STAT3, described by Dr. Rosell's group as a main cause of targeted drug resistance (10), leads to immune response inhibition by inhibiting endogenous retroviral sequence (ERV) expression. Approximately 8% of the human genome consists of ERVs integrated into the human genome hundreds of years ago, which, in the case of some tumors, can be re-expressed, leading to innate antiviral immune response activation through pattern recognition receptor (PRR) (14) activation. STAT3 activates the enzyme DNA (cytosine-5)-methyltransferase 1 (DNMT1), methylating the promoter region of some PRRs as dsRNA-sensing proteins (RIG1) (15). Drugs inhibiting DNMT1, such as 5-azacytidine (16), the administration of vitamin C, a co-factor of TET1 (12), or concomitant radiotherapy that activates response through activation of PRRs (RIG1, TLR3, MAVS) (17), are synergistic with anti PD-1 drugs.

Dr. Rosell also commented on the role of IKKε as a crucial negative regulator of T cell activation and as a potential target for tumor immunotherapy. In melanoma, IKKε promotes nuclear factors of activated T cell (*NFATc1*) phosphorylation and inhibits T cell responses (18). IκB kinase (*IKK*)-related kinases Tank-binding kinase-1 (*TBK1*) and IKKε both promote KRAS-driven tumorigenesis by regulating autocrine CCL5 and interleukin (IL)-6. The inhibition of JAK/TBK1/IKKε has activity against KRAS mutant models (19). TBK1/IRF3 signaling is activated in response to STING, a molecule previously commented by Dr. Gajewski, finally leading to type I IFN induction.

The last talk from this table was performed by Dr. Wellbrok from the Manchester Institute. She commented on her recent publication about MITF as a mechanism of resistance to BRAF inhibitors (20), putting it in context with immunotherapy resistance and its role in the activation of re-expression of ERV sequences. Nelfinavir, an antiretroviral drug, is also a MITF inhibitor that, in combination with BRAF inhibitors, reverses the resistance in melanoma models, in addition, it is also active in NRAS mutated tumors. On the other hand, MIFT regulates the expression of HERV-K, an endogenous retrovirus, in melanoma. Just as Dr. Rosell commented, Dr. Wellbrok also emphasized the role of TBK1 and IKKε for response to immunotherapy. TBK1 and IKKε are activated by the expression of endogenous retroviruses through transmembrane receptors, TLRs, and cytosolic receptors, RIG1/MDA-5, that finally result in the expression of type I Interferon (21).

Novel techniques on cancer research

During this session three investigators from Spanish groups commented on novel techniques for analysis of cfDNA.

Nowadays, the analysis of cfDNA is one of the most relevant fields of research, since it allows molecular analysis of the tumor and molecular monitoring, obviating tissue biopsies that can be risky or uncomfortable for patients.

Dr. Leticia de Mattos-Arruda, who previously worked at the CRUK-Cambridge Institute, and is currently working at the Vall D'Hebron Institute of Oncology, Barcelona, presented her research on Massive Parallel Sequencing or NGS for analysis of cfDNA in both plasma and cerebro spinal fluid (CSF) (22). She also presented unpublished data demonstrating that the mutational landscape across disease evolution reveals challenges posed by tumor heterogeneity. She discussed different techniques for characterizing cfDNA as targeted sequencing, exome sequencing and WES, as well as their role for the clinical follow up of cancer patients. In this regard, Dr. Molina from Hospital Universitari Quiron-Dexeus also commented on the development of a panel using NGS, called SiRe, which covers 568 mutations on six genes (*BRAF*, *CKIT*, *PDGFR*, *NRAS*, *KRAS* and *EGFR*) with a lower limit of detection of 0.01%. He explained that, since mutated alleles in cfDNA might be below 1%, NGS must be narrowed to target only clinically relevant genes. When the panel was evaluated in baseline samples and compared with results of a Taqman-derived assay on tumor tissue, SiRe had a sensitivity of 79% and a specificity of 100% (23).

Dr. Karachaliou from the Dr. Rosell Oncology Institute's Translational Cancer Research Unit, in Barcelona, explained the results from the collaboration with Dr. Wurdinger in the Netherlands using circulating platelets as the source of tumoral RNA. She explained that blood platelets interacting with tumor cells help tumor growth and dissemination. This interaction affects the expression of relevant genes in tumor cells, but also alters the RNA profile of blood platelets (24,25). Cancer cells and the tumor microenvironment release signals that ultimately activate platelet surface receptors, induce specific splicing of pre-mRNAs in circulating platelets, and transform normal platelets to tumor educated platelets (TEP) (26). The combination of these splicing events, as well as the capacity of platelets to ingest circulating mRNA, provides TEPs with a dynamic RNA repertoire that can be used for cancer diagnostics. TEPs can also release a large amount of cytokines and growth factors that act as tumor promoters (27,28). In 2011, two studies analyzed RNA biomarkers in platelets from xenograft models (29) and cancer patients (30). Using reverse transcription-polymerase chain reaction (RT-PCR), they were able to examine the three most common variants of the anaplastic lymphoma kinase (ALK) gene rearrangements with the echinoderm microtubule associated protein-like 4 (EML4), as well as the MET exon 14 skipping mutations, in platelets from non-small cell lung cancer (NSCLC) patients (31,32). They demonstrated that by applying RT-PCR in RNA extracted from platelets of ALK rearranged NSCLC patients, they can detect the EML4-ALK fusion with a sensitivity of 65% and a specificity of 100% (31). In addition, platelet-derived RNA can be used for the real-time monitoring of ALK rearranged NSCLC patients treated with specific ALK inhibitors (31). Also, Thomas Wurdinger's group has demonstrated that TEPs can be successfully used for the detection of cancer among healthy individuals, and for identifying both the location of the primary tumor, as well as the genetic driver alteration among cancer patients (26). Specifically, they found that sequencing the mRNA from TEPs, distinguishes cancer patients from healthy individuals with 96% accuracy. Indeed, the mRNA profile of TEPs was distinct from the profile of healthy donors platelets, with increased TEP mRNAs enriched for biological processes, like vesicle-mediated transport, and cytoskeletal protein binding and decreased TEP mRNAs were involved in RNA processing and splicing (26). With 71% accuracy, Wurdinger and his team were able to correctly identify the location of the primary tumor among six different tumor types, while, with surrogate TEP mRNA onco-signatures, they were able to distinguish if NSCLC, breast or colon cancer patients carried specific genetic alterations (26). In summary, TEPs have several clinical applications for different types of tumors. They can be used as a diagnostic to provide information on the molecular composition of the primary tumor, to predict response to therapy and to monitor the treatment. Their potential as a new cancer-screening test merits further research.

Guess labs

The first talk was presented by Dr. Seoane from Vall D'Hebron Institute of Oncology, Barcelona. He explained the role of TGF-beta on tumor development and as a putative therapeutic cancer target. During tumor progression, TGF beta becomes an oncogenic factor inducing proliferation, angiogenesis, invasion, and metastasis, as well as suppressing the anti-tumoral immune response. He explained that the precise understanding

of the role of TGF in oncogenesis is required in order to design optimal therapeutic approaches and select the patient population that may benefit from an anti-TGF-beta therapy. He reviewed the rationale for evaluating TGF-beta signaling inhibitors as cancer therapeutics, and the progress made in the preclinical and clinical testing of anti-TGF-beta compounds. Tumor cells escape the growth inhibitory effects of TGF-beta by accumulating mutations in components of the TGF-beta signaling as the Smad receptors or loss of mediators of the TGF-beta cytostatic responses. Tumor cells undergo epithelial to mesenchymal transition (EMT) and evade the immunosuppressive environment in response to TGF-beta becoming also more invasive. TGF-beta is involved in a metastasis process increasing the extravasation of tumor cells to distant organs through the endothelial cells (33).

The second speaker was Dr. Puig from the Dermatology Department of the Hospital Clinic in Barcelona. She spoke about how melanoma susceptibility could also have a correlation with melanoma prognosis. Inherited genetic factors may modulate clinical outcome in melanoma. Previous studies have identified several genes and single nucleotide polymorphisms (SNPs) implicated in melanoma susceptibility that modulate melanoma outcome, such as, melanocortin 1 receptor (*MC1R*), the master regulator gene of human pigmentation, and the cyclin dependent kinase inhibitor 2A (*CDKN2A*), that codifies two proteins that work as tumor suppressors, p16 and p14ARF, regulating the cell cycle. *MC1R* is highly polymorphic and some of its functional variants are associated with increased risk of melanoma development and survival (34). Moreover, *MC1R* germinal mutation is associated with a higher somatic mutation burden (35), and it could be a predictive factor of response to immunotherapy with checkpoint inhibitors. Conversely, *MC1R* mutation is associated with resistance to BRAF inhibitors (36). The germinal mutation of *CDKN2A* increased the risk not only of melanoma, but also of other tumors, such as, lung, breast and pancreatic cancers (37), underlining its role in cancer development.

Dr. Vaque from Hospital de Valdecilla in Santander (Spain) explained his work using NGS analysis for three different types of cutaneous cancers: cutaneous T-Cell lymphoma (CTCL), metastatic melanoma (MM) and Merkel cell carcinoma (MCC). To this end, Dr. Vaque's group designed specific cancer-type approaches, consisting of a combination of NGS techniques used alongside other tools commonly employed in diagnosis, such as, immunohistochemistry (IHC), and pre-clinical/clinical research (i.e., patient-derived xenograft *in vivo* models and a clinical trial respectively). For CTCL they identified important mechanisms of tumorigenesis and progression of CTCL regarding PLCG1-downstream signaling as activating mutations in PLCG1, NFKB (CARD11 and RELB) and JAK/STAT (38,39), as well as amplifications in PRKCQ (40). For MM they developed a targeted NGS approach (exons from 217 genes) that enabled the rapid detection (15 days) of important mechanisms of MM disease, as well as case-specific combinations of targeted inhibitors, tested *ex vivo* and *in vivo*, that proved to be more effective when used in appropriate mutational backgrounds (41). For MCC, despite important genetic differences between MCPyV+ and MCPyV– cases, using WES, they uncovered that the MCC tumor can develop similar mechanisms of disease (42). Out of these, P-CREB and P-STAT3 were significantly associated with a worsened prognosis. By means of molecular characterization, Dr. Vaque's group provides novel approaches that can enable the development of specific diagnostic tools, as well as guide specific therapies for CTCL, MM and MCC.

Dr. Escors, from Navarra Biomed, Pamplona (Spain), spoke about the molecular mechanisms of the PD-1/PD-L1 pathway. Anti-PDL1/PD1 therapies are based on the reactivation of tumor-infiltrating T cells by interfering with PDL1-PD1 interactions between cancer cells and T cells. PD1 interactions present on the T cell surface, with its PDL1 ligand expressed by tumor cells, are strongly inhibitory. PDL1-PD1 engagement stops the intracellular signaling pathways that depend on the recognition of the tumor antigen by the T cell receptor (TCR). Dr. Escors explained that in non-neoplastic conditions, the TCR binds to the antigenic peptide complex associated with MHC molecules (pMHC) present on the surface of the antigen presenting cell; then, through phosphorylation, the engaged TCR recruits and activates a collection of intracellular signaling molecules which includes kinases (LCK, ZAP70, PI3K) and phospholipases (PLC-gamma1). These molecules amplify the signal transduction by activating other pathways, including MAP kinases and calcium-dependent signalling. All these molecules comprise the signalosome of the TCR, which regulates proliferation, production of pro-inflammatory cytokines and survival through anti-apoptotic pathways, such as, AKT-mTOR. However, when antigen presentation takes place in the presence of PDL1-PD1 interactions, PD1 interferes with the TCR signalosome

using two mechanisms. Firstly, PD1 recruits SHP1 and SHP2 phosphatases that dephosphorylate ZAP70 and PI3K. Secondly, PD1 induces the transcriptional upregulation of CBL ubiquitin ligases. These ligases ubiquitylate the TCR intracellular domains (alpha and beta chains, and CD3 molecules), causing their internalization and degradation (43). Interfering with PDL1-PD1 interactions with antibodies, or any other means, circumvents these suppressive mechanisms, allowing the T cell to recognise tumor antigens and exert cytotoxicity towards tumor cells.

Dr. Alvarez from Hospital Marques de Valdecilla, Santander (Spain), explained the results of her own research on the development of a DC vaccination for melanoma treatment. The use of DC as cancer vaccines is a form of specific immunotherapy that can stimulate both $CD4^+$ and $CD8^+$ T-cells. However, several factors contributed to the limited success of previous studies using DC vaccination in cutaneous melanoma, such as the induction of regulatory and suppressor T cells, the incomplete maturation and activation of DC, or the induction of Th2 cytokine profiles. In this regard, the intracellular bacterial pathogen, Listeria monocytogenes (*Listeria*), supposes an attractive tool for cancer therapy since it induces Th1 cytokines with prevalence of $CD8^+$ T cell immune responses, localizes within melanocytes and causes apoptosis. Dr. Alvarez described that a DC vaccine loaded with 91–99 peptide of listeriolysin O (LLO), the main virulence factor of *Listeria*, prevents adhesion and dissemination of cutaneous melanoma. Vaccination with DC-LLO_{91-99} causes reprogramming of melanoma's DC antigen presentation, promoting MHC class I, while diminishing MHC class II presentation. This DC reprogramming induces anti-neoplastic Th1 cytokines, interferon gamma (IFN-γ) and IL-12, expansion of antigen and melanoma specific $CD8^+$ T-cell immune responses and reduction of regulatory $CD4^+CD25^+FoxP3^+$ T-cells, responsible for melanoma immune tolerance. Dr. Alvarez proposed DC-LLO_{91-99} vaccination as an adjuvant therapy for cutaneous melanoma that pre-conditions the vaccination site with a potent recall antigen, such as $LLO_{91-99,}$ or as a combination therapy that improves the positive signals between DC and T cells (44).

Translational research in Spanish groups

Dr. Gonzalez-Cao from Dexeus-Quiron Institut in Barcelona, Spain, presented the approved project of a clinical study on durvalumab (an anti PD-L1 antibody) for cancer treatment in HIV infected patients (EUDRACT: 2016-004524-38). The study is a phase II trial with the main objective of demonstrating the feasibility of cancer treatment using anti PD-L1 drugs in HIV infected patients. HIV patients are quite often excluded from cancer clinical trials and, usually, cancer patients with HIV infection have less access to cancer therapies throughout different countries (45). Treating HIV infected patients with anti PD-1/PD-L1 drugs may not only be safe, but could also be therapeutic for the HIV infection. Most of the viral reservoir during chronic HIV infection is on CD4 T cell PD-1 positive, as well as the inactivation, with the expression of PD-1 on CD8 T cells during chronic infection, preventing HIV elimination (46,47). The secondary objective of this clinical trial is to analyze the effect of durvalumab on HIV reservoir.

Dr. Aleix Prat presented the results of NanoString Ncounter as a predictive tool for anti PD-1 treatment. He started explaining the results from a previous work of Dr. Ribas *et al.* using NanoString for prediction of anti PD-1 treatment in melanoma and other tumors. They used a custom 680-gene set on the NanoString nCounter platform. An "interferon-gamma" (IFN-γ) signature was developed in a discovery set of 19 melanoma patients treated with pembrolizumab and was later complemented with an "expanded immune" signature. These signatures were later validated in patients with melanoma, head and neck, squamous cell carcinoma and gastric cancer. In the melanoma validation set, the IFN-γ and the expanded immune signatures were significantly correlated with response and progression free survival (48). Recently, Dr. Prat led a collaboration between NanoString technologies and Merck in order to define a diagnostic test for predicting pembrolizumab activity. He developed seven signatures that were associated with progression free survival in 61 patients treated with anti PD-1, independent of the type of cancer, biopsy, time of tumor biopsy and type of anti-PD1 therapy using the NanoString PanCancer Immune panel (49).

Conclusions

In Spain, a high scientific level of translational research in melanoma will lead to real progress for the treatment and cure of melanoma patients. Coordination and collaboration between basic and clinical researchers is the key for achieving the needed knowledge for a successful melanoma treatment.

The next GEM Translational Meeting will be held in Pamplona (Navarra, Spain) and we hope it will be an important meeting point between basic and clinical researchers.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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