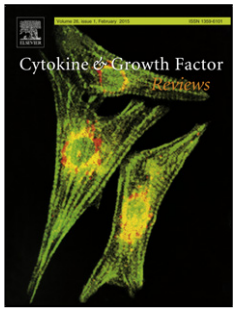
Anticancer innovative therapy: Highlights from the ninth annual meeting



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Anticancer Innovative therapy: Highlights from the ninth annual meeting

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Abstract

The Ninth Annual Conference of "Anticancer Innovative Therapy", organized by Fondazione IRCCS Istituto Nazionale dei Tumori di Milano (Fondazione IRCCS INT) and hosted by Hotel Michelangelo, was held in Milan on 25 January 2019. Cutting-edge science was presented in two main scientific sessions: *i*) pre-clinical evidences and new targets, and *ii*) clinical translation. The Keynote lecture entitled "Cancer stem cells (CSCs): metabolic strategies for their identification and eradication" presented by M. Lisanti, was one of the highlights of the conference.

One key concept of the meeting was how the continuous advances in our knowledge about molecular mechanisms in various fields of research (cancer metabolism reprogramming, epigenetic regulation, transformation/invasiveness, and immunology, among others) are driving cancer research towards more effective personalized antineoplastic strategies. Specifically, recent preclinical data on the following topics were discussed: 1. Polycomb group proteins in cancer; 2. A d16HER2 splice variant is a flag of HER2 addiction across HER2-positive cancers; 3. Studying chromatin as a nexus between translational and basic research; 4. Metabolomic analysis in cancer patients; 5. CDK4-6 cyclin inhibitors: clinical activity and future perspectives as immunotherapy adjuvant; and 6. Cancer stem cells (CSCs): metabolic strategies for their identification and

eradication. In terms of clinical translation, several novel approaches were presented: 1. Developing CAR-T cell therapies: an update of preclinical and clinical development at University of North Carolina; 2. Vγ9Vδ2 T-cell activation and immune suppression in multiple myeloma; 3. Predictive biomarkers for real-world immunotherapy: the cancer immunogram model in the clinical arena; and 4. Mechanisms of resistance to immune checkpoint blockade in solid tumors. Overall, the pre-clinical and clinical findings presented could pave the way to identify novel actionable therapeutic targets to significantly enhance the care of persons with cancer.

1. Introduction

The Ninth Annual Conference on "Anticancer Innovative therapy", organized by Fondazione IRCCS Istituto Nazionale dei Tumori of Milano (Fondazione IRCCS INT), was held in Milan (Italy) on the 25 January 2019. Leading international experts in the fields of immuno-oncology, epigenetic mechanisms controlling tumor proliferation/survival, cancer cell signaling and dysregulated cancer metabolism provided new insights that aim to define more effective therapeutic opportunities. The increasing knowledge about the molecular mechanisms responsible for neoplastic transformation/invasiveness, the role of the epigenetic landscape in defining both chromatin architecture and gene expression profile in cancer cells, the immunological mechanisms regulating tumor-host interaction and the effects of metabolic reprogramming in cancer cell biology will allow researchers and clinicians to identify and apply new, more effective anti-cancer therapeutic options.

To date, immuno-biotherapy is a continuously evolving treatment modality integrated into the treatment algorithms of several different oncotypes. Furthermore, elucidating the resistance mechanisms against anti-tumor therapies and then exploiting novel knowledge of cancer vulnerabilities will ultimately improve cancer therapy and patient survival.

Session I of the conference comprised the following topics: 1) Polycomb group proteins in cancer; 2) d16HER2 splice variant as a flag of HER2 addiction across HER2-positive cancers; 3) the role of chromatin as nexus between translational and basic research; 4) metabolomic analysis in cancer patients; 5) CDK4-6 cyclin inhibitors: clinical activity and future perspectives as immunotherapy adjuvant. Moreover, during this session, the invited keynote lecture was presented by Michael Lisanti who illustrated new metabolic strategies for the identification and eradication of Cancer Stem Cells (CSCs) in breast cancer. Session II included: 1) the development of CAR-T cell therapies: an update of preclinical and clinical development at University of North Carolina; 2) Vγ9Vδ2 T-cell

activation and immune suppression in multiple myeloma; 3) predictive biomarkers for real-world immunotherapy: the cancer immunogram model in the clinical arena; and 4) mechanisms of resistance to immune checkpoint blockade in solid tumors.

Finally, two selected abstracts from young researchers were presented on the following topics: 1) BCL6 as a novel target of triple negative breast cancer stem cells; 2) the capacity of coated cationic liposomes entrapping mir-660 to inhibit tumor growth in patient-derived xenograft lung cancer models. Overall, the latest achievements in the most innovative areas of cancer research were presented and critically discussed.

2. SESSION I: Preclinical evidences and new targets

2.1 Polycomb group proteins in cancer

Adrian Bracken (Trinity College Dublin, Ireland) offered a wide overview on chromatin modifier proteins with a focus on Polycomb group proteins (PCG). In particular, the process through which transcription factors have access to DNA is regulated by a set of chromatin-remodeling machines that alter nucleosome position and structure. The orchestrated regulation of chromatin assembly is critical for gene expression, as it determines its accessibility to specific transcription factors in a precise, sequential order. Chromatin remodelers are ATP-hydrolyzing machines specialized in restructuring, mobilizing or ejecting nucleosomes, which allow exposure of the DNA in the chromatin to be regulated. As cancer is a disease that can be driven by epigenetic changes, it is not surprising that chromatin remodelers are among the most frequently mutated enzymes. PCG are often in multi-protein complex with different functions in the economy of chromatin metabolism. In particular, the SET domain of the catalytic subunit EZH2 is in charge of methyl group deposition at lysine 27 of histone H3 (to produce H3K27me3). It requires the presence of 2 additional proteins, embryonic ectoderm development (EED) and Suppressor of Zeste 12 (SUZ12). Deregulated H3K27me3 levels have been demonstrated in a variety of human cancers (Morin, Mendez-Lago et al. 2011, Cromer, Starker et al. 2012, Hodis, Watson et al. 2012).

A decade ago, acquired EZH2 mutations were identified in lymphoma and myeloid neoplasms. In lymphoma, a heterozygous missense mutation at amino acid Y641, within the SET domain, was identified with high-throughput transcriptome sequencing. In myeloid neoplasm, mutations have been described in poor prognosis myelodysplasia–myeloproliferative neoplasms, myelofibrosis, and various subtypes of myelodysplastic syndromes (Ernst, Chase et al. 2010, Nikoloski, Langemeijer et al. 2010). The therapeutic modulation of epigenetic marks has led to improvements in the treatment of some cancers, for example, by the use of the DNA

demethylating agents azacitidine and decitabine for myelodysplastic syndrome (Fenaux, Mufti et al. 2009, Fenaux, Mufti et al. 2010) and the HDAC inhibitor suberoylanilide hydroxamic acid (vorinostat) for cutaneous T-cell lymphoma (Olsen, Kim et al. 2007). EZH2 inhibition or depletion has been reported to be effective for treating different type of cancers, and several selective inhibitors have been designed to this aim. In particular, GSK126, a selective inhibitor of EZH2, is now in clinical trial. However, in the attempt to consider EZH2 inhibitors in clinical practice, identification of secondary EZH2 mutations that cooperate to confer resistance to these inhibitors must be taken into account, as the possibility exists that acquired resistance to EZH2 inhibitors could occur also in patients (Baker, Nerle et al. 2015, Gibaja, Shen et al. 2016). Based on this broad picture, it is clear that a deep understanding of epigenetic mechanisms that govern basic cellular functions is of fundamental importance for developing more specific, targeted and effective therapies.

2.2 The d16HER2 splice variant is a flag of HER2 addiction across HER2-positive cancers

Serenella M. Pupa (Department of Research, Fondazione IRCCS INT, Milan, Italy) provided the audience with the latest updates on the breast cancer (BC) subtype characterized by the amplification/overexpression of the HER2 receptor (making up 15-20% of BC) (Prat and Perou 2011). In particular, her talk addressed the pathobiological roles displayed by one of the HER2 splice variant, denominated d16HER2, in HER2-positive (HER2+) BC. Although innovative therapeutic options that specifically target HER2 have been introduced and have revolutionized HER2+ disease outcome, about 50% of patients with HER2+ BC are intrinsically resistant or have acquired resistance to these new biodrugs (Loibl and Gianni 2017), implying the need to increase our knowledge about HER2 biology. Evidence suggests that the co-existence of the full-length, wild-type (WT) HER2 oncoprotein (WTHER2) together with altered forms of HER2, such as carboxy-terminal–truncated fragments (Zagozdzon, Gallagher et al. 2011), activating mutations (Weigelt and Reis-Filho 2013), or splice variants (Jackson, Browell et al. 2013), significantly increases the heterogeneity of HER2+ disease, affecting its biology, clinical course, and treatment response (Chen and Weiss 2015). In this context, pre-clinical studies performed in proper transgenic (tg) mouse models provided the first direct evidence that the mammary-specific expression of rodent (HER2/*neu*) as well as distinct forms of human *HER2* genes induced spontaneous tumors development only when coupled with in-frame activating deletions or insertions of cysteine residues within the WTHER2 extracellular domain (HER2/ECD) (Ursini-Siegel,

Schade et al. 2007). In particular, previously reported findings have clearly demonstrated that the imbalance of cysteines plays a crucial role in regulating the catalytic/oncogenic activity of HER2 (Chan, Muller et al. 1999), by activating the receptor as a consequence of constitutive homodimerization through the formation of stable intermolecular disulfide bridges (Chan, Muller et al. 1999, Siegel, Ryan et al. 1999). Along with other groups, Pupa and colleagues reported that HER2+ BC, together with WTHER2, constitutively express the d16HER2 splice variant, structurally characterized by the in-frame deletion of exon 16 in the juxtamembrane region of HER2/ECD fragment. This deletion promotes the elimination of two crucial cysteines and, in turn, the formation of constitutively-activated d16HER2 homodimers (pd16HER2D) on the tumor cell surface (Kwong and Hung 1998, Siegel, Ryan et al. 1999, Castiglioni, Tagliabue et al. 2006, Mitra, Brumlik et al. 2009). Generation and then comparison of specific transgenic cell lines for the human d16HER2 variant (Marchini, Gabrielli et al. 2011) and the WTHER2 isoform (Finkle, Quan et al. 2004) revealed a significantly shorter tumor latency period and a higher tumor incidence in the d16HER2 line, clearly pointing at the candidacy of d16HER2 as the oncogenic "driver" isoform of the HER2 gene (Castagnoli, Iezzi et al. 2014). Additionally, therapeutic experiments performed in parallel in the two transgenic mouse lines with the humanized monoclonal antibody trastuzumab (T), the gold standard care for HER2+ tumors, provided evidence of a significantly higher susceptibility of lines containing d16HER2 than those with WTHER2 to the biodrug (Castagnoli, Iezzi et al. 2014). In the same study, novel important insights into the functional relationship between the splice variant d16HER2D and activated SRC (pSRC) were provided both in pre-clinical and clinical settings, allowing us to consider intratumor pSRC expression as a surrogate marker of d16HER2D expression in HER2+ BC (Castagnoli, Iezzi et al. 2014). Further, a Gene Set Enrichment Analysis (GSEA) of the whole transcriptome of human HER2+ BC specimens stratified for high *versus* low levels of pSRC expression, the intra-tumor mirror of pd16HER2D expression, showed significantly enrichment in hypoxia, tumor metastasis, and cell motility gene pathways only in high-expressing pSRC/d16HER2 BC. This strongly suggests that d16HER2 is a key molecule involved in HER2-driven aggressiveness and stemness. Accordingly, in recent studies, Pupa and colleagues demonstrated that the enrichment of d16HER2 expression *versus* its wild-type counterpart (WTHER2) significantly influences HER2+ BC-initiating cells (BCIC) properties (Castagnoli, Ghedini et al. 2017). Further, her team provided evidence of a significant up-regulation of glucose metabolism in HER2+ BC models enriched in the pd16HER2/pSRC signaling axis *versus* those characterized by low axis expression levels, thus implying an accelerated energy consumption in

high pd16HER2/pSRC–expressing BC (Castagnoli, Iorio et al. 2019). Altogether, the findings indicate that d16HER2 expression/activation strongly reflects the status of HER2 oncogenic signaling dependence, which has been defined as a "HER2 addiction" (Alajati, Sausgruber et al. 2013, Turpin, Ling et al. 2016, Castagnoli, Iorio et al. 2019) and represents a novel and potentially clinically useful biomarker (Castagnoli, Ladomery et al. 2019). Lastly, to overcome the difficulty of intra-tumor detection of the d16HER2 isoform at the protein level, Pupa and colleagues recently established a novel mRNA bright-field *in situ* hybridization (ISH) technique to score and discriminate intra-tumor expression of d16HER2 mRNA from the WTHER2 mRNA. Applied to HER2+ BC and gastrointestinal (GC) malignancies, this technique allowed them to demonstrate the existence of outliers with high d16HER2 mRNA scores that only occurred in HER2+ GC (Volpi, Pietrantonio et al. 2019). Of note, a significant association between having a high d16HER2 score and prolonged progression free survival was found also in HER2+ GC treated with T, strongly suggesting that d16HER2 could be clinically investigated as a marker of T susceptibility in several HER2-driven cancers (Castagnoli, Ladomery et al. 2019).

2.3 Studying chromatin as a nexus between translational and basic research

The highly-regulated packaging of the genome into chromatin represents the basis of epigenetic regulation, and alterations at the epigenetic level contribute to cancer development and progression. **Marcus Buschbeck (Josep Carreras Leukaemia Research Institute, Campus ICO, Germans Trias I Pujol, UAB, Barcelona, Spain)** illustrated data obtained in his lab aimed at understanding chromatin regulation at the mechanistic level. Focusing on the age-related hematopoietic stem cell defect myelodysplatic syndrome (MDS) and its progression to acute myeloid leukemia (AML), the speaker showed that RING1A, the catalytically active subunit of the Polycomb repressive complex 1 (PRC1), is overexpressed in high-risk patients with MDS. This highlights the key role of this epigenetic regulator in the pathogenesis of MDS. However, RING1A pharmacological inhibition affects the stem cell compartment in both MDS patients and healthy donors, hampering its clinical applicability (Palau, Garz et al. 2017). The Buschbeck group applies genetic screening methods (Fellmann, Hoffmann et al. 2013) and cell culture models to identify chromatin regulators able to increase the response to the nucleoside-analogue azacitidine. Although azacitidine is the current treatment of choice for high-risk patients with MDS who are not eligible for allogenic hematopoietic stem cell transplantation, the success is limited by the occurrence of primary and secondary therapy resistance (Diesch, Zwick et al. 2016). Dr. Buschbeck

presented his most recent promising results showing that using targeted small molecule inhibitors to inhibit histone-modifying enzymes can increase the cytotoxic response of AML cells to azacitidine. If further validated *in vivo*, the use of such inhibitors would provide the rationale for the development of a combinatorial treatment. His lab is also actively involved in understanding the function and regulation of a group of macroH2A histone variants in embryonic development and cancer. The regulated deposition of histone variants into specific chromatin regions can directly influence gene transcription due to their ability to alter nucleosome stability and chromatin organization (Buschbeck and Hake 2017). Unlike other histones, the structure of macroH2As is composed of two C-terminal domains, an unstructured linker that contributes to higher-order chromatin architecture (Douet, Corujo et al. 2017, Kozlowski, Corujo et al. 2018), and a globular macrodomain that is a binding module for the NAD+–derived metabolite ADP ribose (Posavec, Timinszky et al. 2013). As the isoform macroH2A1.1 is also able to bind ADP-ribosylated proteins, it consequently binds and inhibits the stress sensor PARP1 (Hurtado-Bages, Guberovic et al. 2018). Thereby, macroH2A1.1 can limit PARP1-dependent chromatin remodeling after DNA damage (Kozlowski, Corujo et al. 2018). As PARP1 is the major NAD+ consuming enzyme, inhibition by macroH2A1.1 impacts global NAD+ metabolism (Posavec Marjanovic, Hurtado-Bages et al. 2017). In this context, Buschbeck and colleagues are investigating whether the function of the linker in 3D chromatin architecture and of the macrodomain in metabolite-binding are coupled. This would further define the role of macroH2A histone variants in connecting metabolic cellular clues to heterochromatin architecture.

2.4 Metabolomic analysis in cancer patients

Andre Wegner (Technische Universität [TU] Braunschweig, Germany) exemplified the potential of the metabolomic analysis in cancer research. Metabolic reprogramming is known to be an important hallmark of cancer cells (Hanahan and Weinberg 2011). It is often the result of alterations in the upstream compartments (genome, transcriptome, proteome) and directly affects the phenotype of a biological system (Cantor and Sabatini 2012). Uncovering the mechanisms underpinning the metabolic alterations opens novel therapeutic opportunities. In particular, analyzing changes in the total pool of metabolites, i.e. metabolomics, can be exploited to discover novel disease biomarkers, although it only provides a static picture about metabolism. In contrast, analyzing metabolic fluxes can better elucidate disease mechanisms and identify possible therapeutic targets. To this end, the use of stable isotope (i.e. ^{13}C)–labelled metabolites

as metabolic substrates in the context of ^{13}C-metabolic flux analysis (^{13}C-MFA) is a powerful tool for following specific metabolites production and consumption (Lane, Fan et al. 2009). The mass isotopomer distribution (MID) can then be evaluated by mass spectrometry (MS) for each metabolite with a ^{13}C-label incorporated, and the labeling patterns are analyzed to trace specific pathways and calculate metabolic fluxes.

Dr. Wegner also presented data on lung cancer cell metabolism. In particular, they evaluated the metabolism of the lung cancer cell line A549 by incubating cells with [U-^{13}C]glutamine or [1,2-^{13}C]glucose, and subsequently analyzing the MID by gas chromatography MS (GC/MS). Surprisingly, the metabolite N-acetyl-aspartic acid (NAA), known to be the most abundant amino acid in the brain, was produced by these lung cancer cells, and synthesized by the N-acetyltransferase 8-like (NAT8L) enzyme, as confirmed by silencing experiments. Even though the NAA functional role in the context of lung cancer remains unclear, Wegner and collaborators showed that loss of NAA by NAT8L silencing reduced cell proliferation (Weindl, Cordes et al. 2016). NAT8L knockout also had consequences at the metabolic level, such as decreased levels of glycerol-3-phosphate, while NAA overexpression induced increases in aspartic acid and glycerol-3-phosphate. Strikingly, NAA did not show ^{13}C label incorporation when permeabilized cells (Nonnenmacher, Palorini et al. 2017) were cultured with either pyruvate or glutamine that had been ^{13}C-labelled, suggesting that this metabolite is not produced from pyruvate or glutamine in the mitochondria. Further experiments are needed to better elucidate the role of NAA in lung cancer.

2.5 CDK4-6 cyclin inhibitors: clinical activity and future perspectives as immunotherapy adjuvant

Serena Di Cosimo (Department of Applied Research and Technological Development, Fondazione IRCCS INT, Milan, Italy) addressed the key topic of CDK4/6 inhibitors as modulators of immune response for anti-tumor therapies. In particular, and according to the recent literature on this subject, Dr. Di Cosimo reported that CDK4/6 inhibitors are effective in treating advanced hormone receptor–positive, HER2-negative metastatic BC. Combined with endocrine therapy (i.e. the aromatase inhibitor letrozole and the selective estrogen receptor degrader fulvestrant), the CDK4/6 inhibitors palbociclib, ribociclib, and abemaciclib have substantially improved progression-free survival and response rates when used both as the first and advanced lines of treatment for this category of patients. The drugs work by selectively turning off overactive CDK4 and CDK6, which in turn restore the growth-suppressive properties of the retinoblastoma (Rb) protein, a

well-known cell cycle progression checkpoint. As a result, the division cycle in cancer cells is halted, preventing them from proliferating.

Recent work has revealed that the antitumor activity of CDK4/6 inhibitors not only rely on blocking cell division, but also on unleashing the immune system to attack the cancer cells. Different preclinical studies using animal models of BC found that the CDK4/6 inhibitors (i) induce secretion of IFN gamma, which in turn enhances the presentation of tumor antigens to cytotoxic T cells; (ii) reduce the population of immune-suppressing T-regulatory cells in the tumor microenvironment; and (iii) increase the antitumor activity of immune checkpoint–blocking antibodies **(Figure 1)** (Goel, DeCristo et al. 2017, Deng, Wang et al. 2018, Schaer, Beckmann et al. 2018).

INSERT Figure 1

Even though the CDK4/6 inhibitors have been shown to be most effective for metastatic luminal BC, and are only approved for wide use in that type of cancer, evidence shows promising activity also in other forms of cancer, including liposarcoma, non–small-cell lung cancer, mantle cell lymphoma, melanoma, and glioblastoma. Hence, these new findings significantly expand the use of CDK4/6 inhibitors for treatment of other cancers based on an immune-mediated effect. Finally, two studies are currently evaluating CDK4/6 inhibitors and their potential to modulate immune response in women with metastatic and locally advanced BC, who should receive endocrine therapy in combination with immune checkpoint–blocking antibodies pembrolizumab or avelumab, respectively.

2.6 Cancer stem cells (CSCs): metabolic strategies for their identification and eradication

Michael Lisanti (University of Salford, UK) presented the keynote lecture on new possible approaches to counteract CSCs. Mitochondria are known to be the energetic hub of a cell, and their function and homeostasis is fundamental for cell survival. The “reverse Warburg effect” theory states that cancer cell mitochondria oxidatively metabolize nutrients (i.e. lactate, ketones) provided by adjacent stromal cells that are undergoing aerobic glycolysis (Pavlides, Whitaker-Menezes et al. 2009), the so-called “two-compartment tumor metabolism” model (Sotgia, Whitaker-Menezes et al. 2012). This metabolic coupling supports mitochondrial ATP production via oxidative phosphorylation (OXPHOS) in the anabolic cancer cells, promoting a stemness phenotype, metastatic potential, and tumor growth (Martinez-Outschoorn, Peiris-Pages et al. 2017). Given the key role of mitochondrial metabolism in CSC maintenance and drug resistance (De Luca, Fiorillo et al. 2015), Dr. Lisanti’s group was interested in finding new approaches to

counteract CSC via targeting mitochondria. They first demonstrated that mitochondrial proteins (both metabolic enzymes as well as mitochondrial biogenesis-related proteins) are upregulated in mammospheres derived from the MCF7 and T47D BC cell lines (Lamb, Harrison et al. 2014). The pivotal involvement of mitochondria in supporting a stemness phenotype was further confirmed by the detrimental effect of oligomycin A treatment (a known inhibitor of the mitochondrial ATP synthase) on the mammosphere forming efficiency (Lamb, Harrison et al. 2014). Mitochondrial metabolism could therefore be exploited to eradicate CSCs in various tumor types. To this aim, Lisanti and colleagues demonstrated that mitochondrial mass could be used as a metabolic biomarker for stem-like cancer cells. In particular, cells with high mitochondrial mass ("mito-high", as detected by the MitoTracker deep red dye) more closely resembled the CSC features, such as having higher ALDH activity, mammosphere formation capacity, tumorigenic potential, and a chemo-resistant phenotype (Farnie, Sotgia et al. 2015). The possibility to isolate the most therapy-resistant components of a tumor represents a powerful tool to investigate drug sensitivity in the context of personalized cancer treatment **(Figure 2).**

INSERT FIGURE 2

Different classes of FDA-approved antibiotics are known to inhibit mitochondrial biogenesis as an "off-target" effect. Lisanti and collaborators have suggested re-purposing antibiotics (i.e. doxycycline) for CSC eradication as a novel cost-effective cancer treatment approach of broad applicability (Lamb, Ozsvari et al. 2015). Doxycycline treatment has indeed shown the capacity to reduce the mammosphere-forming efficiency of BC cell lines *in vitro*, as well as of CSCs derived from metastatic BC patients (Lamb, Ozsvari et al. 2015). Interestingly, doxycycline has also shown effects on the MCF7 cell metabolism; in particular, it was able to reduce levels of both OXPHOS and glycolysis, transforming the highly energetic cells into quiescent ones.

In the clinical setting, doxycycline treatment has previously shown promising results in MALT lymphoma patients (Ferreri, Ponzoni et al. 2006). Moreover, a phase II clinical trial testing the combination of doxycycline and chemotherapy has been approved for early BC patients at the Pisa University Hospital (Italy). Preliminary results showed reduced CD44 and ALDH1 expression in the BC tissue after doxycycline administration. With the idea to overcome a possible development of antibiotic resistance, Lisanti and collaborators explored several treatment combinations with metabolic inhibitors, aimed at exploiting the metabolic features and vulnerability acquired during adaptation to treatments. For instance, BC cells resistant to doxycycline are characterized by a glycolytic phenotype that can be successfully targeted using vitamin C (aerobic glycolysis inhibitor)

in a synthetic lethal approach able to eradicate these CSCs (De Francesco, Bonuccelli et al. 2017). Recent data from the Lisanti group revealed the existence of a subset of CSCs defined "energetic CSCs", characterized by higher metabolic activity (higher mitochondrial OXPHOS), hyper-proliferation, enrichment in stem cell features (ALDH activity, sphere-forming efficiency, mitochondrial mass), and dependence on a 3D micro-environment. Given these peculiar characteristics, the "energetic CSC" subpopulation can be pharmacologically targeted with OXPHOS inhibitors (i.e. DPI) and CDK4/6 inhibitors (i.e. ribociclib) (Fiorillo, Sotgia et al. 2018). Overall, these novel approaches targeting CSC mitochondria with repurposed drugs pave the way to more affordable as well as highly effective and durable treatments.

3. SESSION II: Clinical translation

3.1 Developing CAR-T cell therapies: an update of preclinical and clinical development at the University of North Carolina

Gianpietro Dotti (University of North Carolina, Chapel Hill, USA) presented his most recent data in CAR-T cell therapy. The engineering of chimeric antigen receptors (CARs) in T-cells has fuelled the rapid generation of tumor-specific cells, increasing the clinical applicability of adoptively–transferred cell therapies. Targeting CD19 via CAR-T cells is highly effective in B-cell malignancies (Hay and Turtle 2017). A similar approach was developed to treat Hodgkin's lymphoma (HL) by targeting CD30. Treatment with CAR-modified T-cells targeting CD30 (CD30.CAR-Ts) without lymphodepletion was found to be safe with preliminary efficacy in patients with relapsed/refractory (r/r) CD30+ lymphomas (Figure 3) (Ramos, Ballard et al. 2017). Prof. Dotti reported the results of a phase 1b/2 trial of CD30.CAR-Ts infused after lymphodepletion with either bendamustine (benda) alone, or a combination of benda and fludarabine (flu), in 24 patients with r/r CD30+ Hodgkin (HL) and non-Hodgkin lymphoma (NHL) who had undergone a median of 7.5 prior lines of therapy. They observed no dose-limiting toxicities, no neurological toxicity, and mild and self-limiting cytokine release syndrome. At the 6-week assessment, 10 patients in the benda/flu cohort had CR (53%), 2 had partial response (11%), 2 had stable disease (11%), and 5 had progressive disease (26%, including all 3 patients treated at DL1). The addition of flu to lymphodepletion also improved T-cell expansion and persistence. The trial showed that autologous CD30.CAR-T cells are extremely well tolerated and can have significant clinical activity in heavily pre-treated patients with r/r HL when the lymphodepleting conditioning regimen includes flu. The retained expression of PD-1 on CD30.CAR-T cells indicates that these cells likely

remain susceptible at the tumor site to the PD-L1 inhibition, suggesting that the next step to further enhance efficacy will require a combination of CD30.CAR-T cells and checkpoint blockades.

INSERT FIGURE 3

3.2 Vγ9Vδ2 T-cell activation and immune suppression in multiple myeloma

Massimo Massaia (Department of Molecular Biotechnology and Health Sciences, Turin, Italy) discussed the main issues in the application of Vγ9Vδ2 T-cell–based immunotherapy in the context of multiple myeloma (MM). Vγ9Vδ2 T-cells are considered as non-conventional, bearing the characteristics of both adaptive and innate immunity. In particular, these cells are able to react against malignant B-cells, and therefore against malignant myeloma cells, and are activated by self-phosphoantigens (pAgs), including the phosphorylated metabolites produced in the mevalonate pathway (i.e. isopentenyl pyrophosphate, IPP) (Castella, Vitale et al. 2011). Given the capacity of zoledronic acid (ZA) to inhibit the mevalonate pathway, this could be exploited to test Vγ9Vδ2 T-cells reactivity. In fact, ZA treatment on antigen-presenting cells (APC) induces the intracellular accumulation and extracellular release of IPP, which is detected by Vγ9Vδ2 T-cells. However, Vγ9Vδ2 T-cells derived from the bone marrow of patients with MM or monoclonal gammopathy of undetermined significance (MGUS) are characterized by a defective reactivity to IPP and are therefore considered anergic. These cells are also PD-1+, in line with the immunosuppressive TME of MM patients. Interestingly, single PD-1 blockade induced the expression of multiple immune checkpoint molecules (ICPs), such as PD-1, TIM-3, and LAG-3, on the MM BM-infiltrating Vγ9Vδ2 T-cells (thereby defined a “super-anergic” state). These results suggest that a more aggressive approach of combining multiple anti-ICP antibodies is the only way to effectively overcome TME immunosuppression. However, the clinical application of this approach is limited by the huge costs and the high toxicities induced by these drugs. Further studies to understand the mechanisms underpinning ICP expression are necessary to be able to rescue Vγ9Vδ2 T-cells from their immune dysfunction (Castella, Melaccio et al. 2018).

3.3 Predictive biomarkers for real-world immunotherapy: the cancer immunogram model in the clinical arena

Giancarlo Pruneri (Fondazione IRCCS INT, Milan, Italy) illustrated the existing predictive biomarkers in the context of immunotherapy. Despite having highly promising therapeutic potential, immunotherapy results in clinical benefit for only a small portion of patients (8–10%). It is therefore clear that a more accurate patient selection is necessary. The abundance of tumor-

infiltrating lymphocytes (TILs) can be considered a read-out of tumor immunogenicity. Indeed, a higher TIL infiltration is detected in the basal-like and HER2+ subtypes of BC (Luen, Virassamy et al. 2016). Moreover, TIL detection has shown prognostic value of response to therapy in TNBC patients in the adjuvant setting (Pruneri, Gray et al. 2016). Recent clinical evidence demonstrate that quantification of stromal TILs in BC patients has a subtype-dependent prognostic role and can be considered as a reproducible biomarker (Loi, Drubay et al. 2019). Interestingly, having a percentage of stromal TILs of >20% in node-negative early-stage TNBC patients correlates with a good prognosis (Loi, Drubay et al. 2019).

Tumors with high prevalence of somatic mutations (>150 mutations) are prone to neoantigen formation (Schumacher and Schreiber 2015), and as such are more immunogenic and show survival benefits after immune checkpoint blockade. The tumor mutational burden (TMB) is therefore considered a promising biomarker, and its detection has been included in clinical trials. For instance, a high TMB demonstrated improved efficacy of nivolumab or nivolumab/ipilimumab treatment in small-cell lung cancer (Hellmann, Callahan et al. 2018) and NSCLC (Hellmann, Nathanson et al. 2018). Interestingly, the efficacy was further improved (higher complete/partial response rate) in patients with both high TMB and PD-L1 positivity (Hellmann, Nathanson et al. 2018), suggesting that the combination of two or more biomarkers could be a more reliable approach to stratify patients.

However, standardized gene panels are needed to introduce TMB detection into the clinical routine. The Oncomine Tumor Mutation Load Assay (ThermoFisher) represents a multi-biomarker next-generation sequencing-based assay useful to profile clinical samples and is able to detect 409 cancer-driver genes. However, Dr. Pruneri stressed that the biggest limitation of this approach is technical: FFPE processing, and in particular the formalin fixation step, is able to induce somatic mutations itself, affecting the interpretation of results. Further optimizations are therefore needed in order to consider TMB as a reliable biomarker.

3.4 Mechanisms of resistance to immune checkpoint blockade in solid tumors

Andrea Anichini (Fondazione IRCCS INT, Milan, Italy) addressed the important question about resistance to immune checkpoint blockade (ICB) in the context of solid tumors. Clinical efficacy of immunotherapy by ICB is often hampered by a number of mechanisms of intrinsic and acquired resistance (Galluzzi, Chan et al. 2018). Several of these mechanisms act by preventing development of anti-tumor immunity at tumor sites and/or by inhibiting effective tumor

recognition and elimination, even when tumor-specific T-cells are present. Dr. Anichini investigated these mechanisms by integrating the assessment of selected immune-related gene signatures with the analysis of immune contexture and of HLA expression at the tumor site. A key aspect of the approach was the comparison of pre- and post-therapy lesions from melanoma patients treated with anti-CTLA-4 (Jerby-Arnon, Shah et al. 2018) and from bladder cancer patients treated with anti-PD-1 antibody (Necchi, Anichini et al. 2018). In the melanoma patients who did not respond to treatment, the post-therapy lesions showed significant reduction of expression of genes involved in T-cell differentiation/function (LCK, GZMB, TBX21), T-cell recruitment (CXCL9, CXCL10), IFN- γ, and STING pathway functions, as well as HLA class I antigen processing and presentation (NLRC5, TAP1, TAP2, PSMB9) (as compared to pre-therapy lesion). This indicates that multiple mechanisms of resistance are acting at the same time in the same lesion. Reduced expression of HLA class I molecules on neoplastic cells, as well as T-cell exclusion from the tumor tissue, were confirmed by immunohistochemistry in pre- and post-therapy lesions from several non-responding patients (Jerby-Arnon, Shah et al. 2018). In bladder cancer patients enrolled in a neoadjuvant trial with anti-PD-1, immune-related gene signature analysis was carried out by comparing pre-therapy biopsies with the post-therapy surgical sample. In non-responding patients, this analysis showed that the therapeutic antibody was effective in the activation of anti-tumor immunity (increased expression of T-cell–related genes, T-cell–recruiting chemokine genes, and IFN-γ–related genes in post-therapy surgical samples). However, these positive effects coexisted with strong promotion of several mechanisms of resistance such as: I) upregulation of inhibitory receptors and ligands genes, II) promotion of IDO1 expression, and III) boost in expression of genes coding for chemokines that recruit myeloid suppressor cells (Necchi, Anichini et al. 2018). Taken together, these results suggest that resistance to immune checkpoint blockade cannot be easily explained by a single dominant effect but instead often results from multiple, co-occurring mechanisms that impair different steps along the cancer immunity cycle.

4. Award for the best abstract

Lastly, two selected abstracts from young researchers were presented.

The existence of CSC represents a big threat for TNBC patient clinical outcome, given the CSC therapy-resistant capacity (Chang 2016). Therefore, the identification of therapeutic approaches that could lead to their eradication is of seminal importance for reducing tumor recurrence and improving patient survival.

Tatiana Volpari (Unit of Immunotherapy and Anticancer innovative Therapeutics, Fondazione IRCCS INT, Milan, Italy) presented novel data on the role of the transcriptional repressor BCL6 in regulating stemness features of TNBC stem cells. In particular, BCL6 overexpression was associated with a worse overall survival in TNBC patients. Moreover, GSEA results highlighted an enrichment in stemness signature genes in TNBC cases that overexpressed BCL6. Among the signaling pathways known to have a role in TNBC stemness, the NOTCH pathway showed a higher enrichment in patients with BCL6–TNBC, using an *in silico* approach. Volpari and colleagues therefore further investigated the role of BCL6 in TNBC stem cells *in vitro*, taking advantage of a highly potent and specific BCL6 inhibitor, the small molecule FX1, which is able to disrupt the recruitment of BCL6 co-repressors, thereby inhibiting BCL6's repressive function on its target genes (Cardenas, Yu et al. 2016). The results obtained point to BCL6 as a key molecule in maintaining proliferation and self-renewal capacity of TNBC stem cells. Moreover, the molecular mechanisms supporting these evidences were investigated, and a novel BCL6 target gene was identified (*manuscript in preparation*).

Lung cancer is the leading cause of cancer-related deaths due to late diagnosis and inadequate therapies (Siegel, Miller et al. 2018). MicroRNAs (miRNAs) are small non-coding RNAs that are de-regulated in cancer and are involved in lung cancer development (Boeri, Pastorino et al. 2012). By targeting multiple transcripts, miRNAs epigenetically regulate fundamental cellular processes such as cell proliferation, apoptosis, differentiation, and migration, strongly indicating that they may function as potential oncogenes or tumor suppressors in cancer development (Iorio and Croce 2012). Due to their ability to simultaneously regulate several cancer-related genes, they represent an interesting therapeutic approach for cancer treatment (Gandellini, Profumo et al. 2011).

Orazio Fortunato (Fondazione IRCCS INT, Milan, Italy) and colleagues have previously demonstrated that miR-660 is downmodulated in plasma and tissues of lung cancer patients, and that re-introducing it reduced lung cancer growth by inhibiting the MDM2-P53 axis (Fortunato, Boeri et al. 2014). They have now developed coated cationic liposomes containing miR-660 mimic (CCL660) and tested their efficacy in established lung cancer PDX mouse models (Moro, Bertolini et al. 2017). Systemic delivery of liposomal miR-660 increased miRNA levels in tumors and significantly reduced tumor growth in two p53 wild-type PDXs without off-target effects. CCL660 administration reduced the number of proliferating cancer cells by inhibiting MDM2 and restoring p53 function as well as that of its downstream effectors, such as p21. Interestingly, inhibition of tumor growth in the p53 mutant PDXs that have a functional p21 pathway was observed following

CCL660 administration. Moreover, to assess the potential role of miR-660 in metastatic dissemination, H460 metastatic lung cancer cells overexpressing miR-660 were intravenously injected into SCID mice. Analysis with PET imaging showed a reduction of the formation of lung cancer nodules, which was confirmed by immunohistochemical analysis in H460-mir-660 cells, suggesting a potential role of mir-660 in the metastatic process. As miRNA accumulation in non-target organs was also observed, a potential for toxic effects of both miRNAs and liposomes was questioned. However, *in vitro* assays demonstrated that miR-660 overexpression did not induce any changes in terms of proliferation or apoptosis in normal cells from either mouse or human. Notably, a phase 1 clinical trial using MRX34 in liver cancers has been halted due to multiple immune-related severe adverse events (Chakraborty, Sharma et al. 2017). To address this, Fortunato and colleagues have now analyzed immune infiltrates and pro-inflammatory cytokine release in immunocompetent mice after single or prolonged CCL660 administration. Interestingly, liposome entrapping synthetic miR-660 had no immunological off-target or acute/chronic toxic effects in immunocompetent mice.

Altogether, these promising results offer a new boost for the development of miRNA-delivering compounds in the treatment of lung cancer that no longer induce immune-related toxic effects.

6. Conclusions

Cancer research is taking important steps forward towards understanding the molecular mechanisms involved in cancer development, maintenance, invasiveness, metastasis, and importantly, the mechanisms underlying anti-tumor therapy resistance. Tumors are no longer considered as merely “composed of cancer cells”; rather, the critical and regulatory role of the tumor microenvironment (TME), comprising stromal cells as well as the anti-tumor/pro-tumor immune system, is becoming increasingly clear. Novel therapeutic approaches should therefore take into account all these aspects to succeed in improving patient care.

During the Ninth Edition of the Annual Congress on “Anticancer Innovative therapy”, experts in the field of immuno-oncology, epigenetics, tumor cell signaling, and cancer metabolism shared their latest knowledge acquired on the roles of i) epigenetics, and in particular, chromatin modifiers, ii) cancer metabolism, iii) cancer stem cells (CSCs), iv) tumor cell signaling, and iv) the immune system. The novel therapeutic approaches presented included epigenetic drugs, cell cycle inhibitors combined with ICB, antibiotics and other off-label drugs, small-molecules active against CSCs, liposome-delivered miRNAs, tumor-specific CAR-T cells, and T-cell–based immunotherapy.

Moreover, important evidence on possible mechanisms of resistance to these innovative therapies were also discussed, in particular with respect to resistance to ICB.

Overall, this conference provided scientists and clinicians with a broad overview of future challenges and hopes for improved cancer treatment in the short term.

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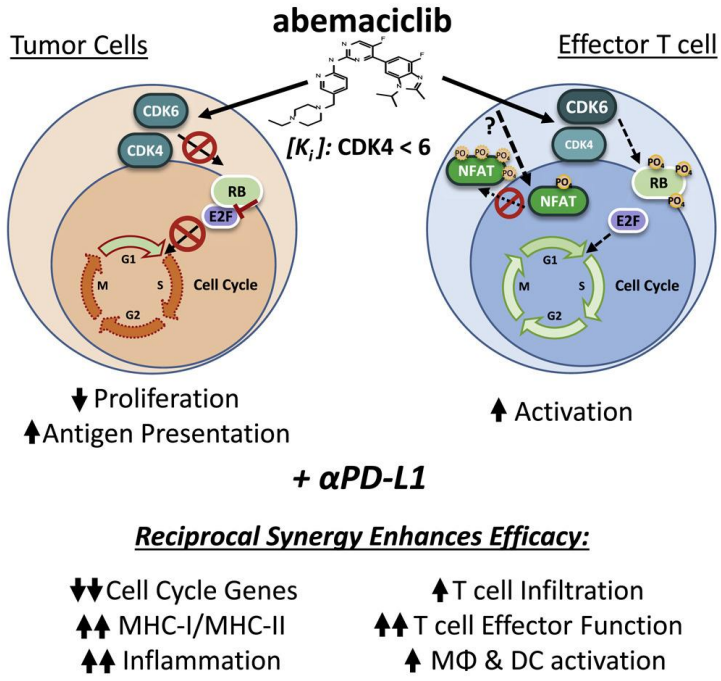

Figure 1. **Immune-modulating effects of CDK4/6 inhibitors**. CDK4/6 inhibitors exert their anti-tumor effects not only by blocking cell division, but also by inducing the immune system. In particular, they enhance antigen presentation capacity of tumor cells and increase T-cell activation, leading to a synergistic effect with anti-PD-L1 therapy (from DA Schaer et al., 2018).

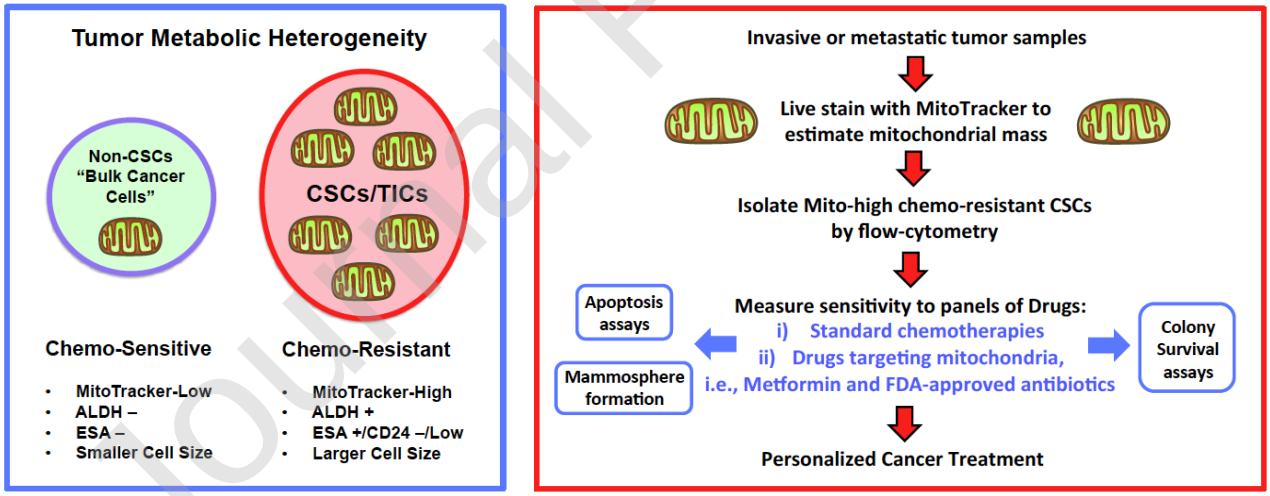

Figure 2. **High mitochondrial mass as a novel marker of chemo-resistant CSCs.** Live MitoTracker staining could be exploited to discern CSCs with high (mito-high) and low (mito-low) mitochondrial mass. Considering the drug-resistant capacity of the mito-high subpopulation, a high mitochondrial mass can be considered a biomarker of chemo-resistant CSCs that can be easily isolated and subjected to drug screening. This approach could therefore represent a novel clinical strategy towards personalized cancer treatment (from Farnie et al., 2015).

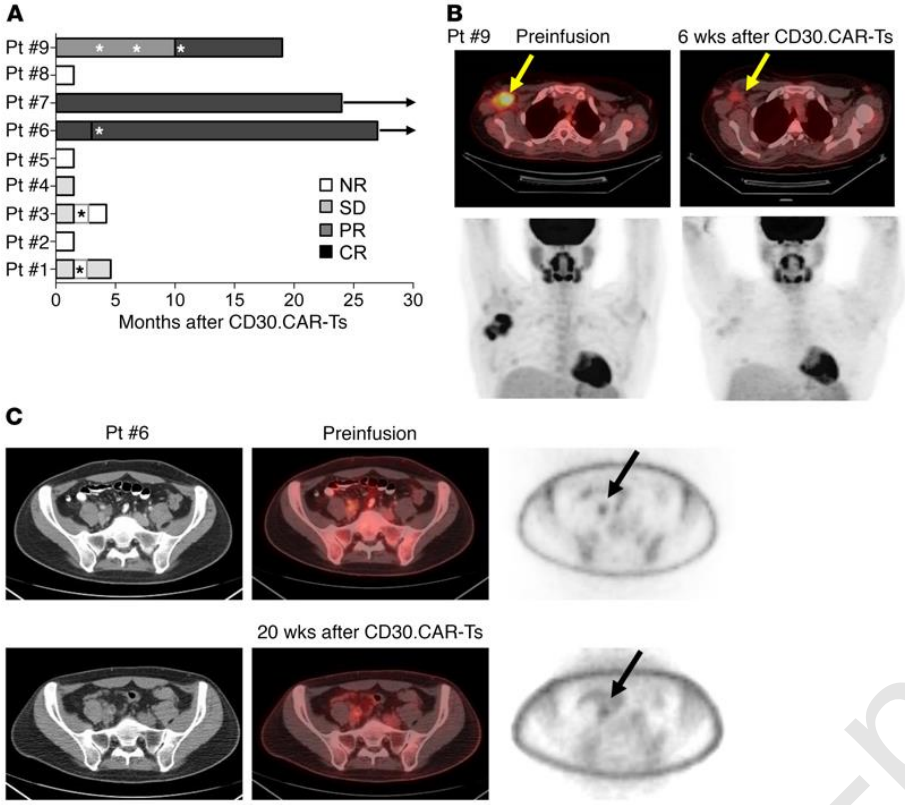

Figure 3. Efficacy of CD30.CAR-Ts in lymphoma patients. Nine patients were infused with CD30.CAR-Ts, and their clinical responses were evaluated by PET. (A) CR, complete response; NR, no response; PR, partial response; SD, stable disease. (B) Six weeks after the first infusion of CD30.CAR-Ts, PET scans showed PR in patient #9, and CR was obtained after the fourth infusion for this patient. (C) CR was observed six weeks after the first infusion of CD30.CARTs also for patient #6, and confirmed 20 weeks later (from Ramos CA et al., 2017).