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TRYPTOPHAN SHORTAGE DUE TO IDO-1 EXPRESSED BY HIGH-DENSITY NEUTROPHILS INDUCE IMMUNE-SUPPRESSION AND PLASMA CELL FITNESS IN MULTIPLE MYELOMA

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Background: There is an increasing interest about the role of amino acid degrading enzymes in cancer immunotherapy. In multiple myeloma (MM), several groups including ours showed that immune-suppression due to arginine starvation is clinically relevant. However, Arginase inhibition or arginine supplementation cannot recover completely T-cell immune dysfunction.
Aims: In the attempt to investigate the role of amino acid deprivation in MM progression, we tested if availability of tryptophan could hamper immune function in the progression from MGUS through MM.
Methods: We first measured the amount of tryptophan and its degrading enzyme 2,3-indoleamine deoxygenase (IDO-1) in sera obtained from bone marrow and peripheral blood of 15 MGUS, 10 smoldering MM (sMM), 15 newly diagnosed and 10 relapsed MM. Second, we evaluated the main cellular source of circulating IDO by western blot and immune fluorescence of high- and low-density neutrophils (LDN), monocytes and neoplastic plasma cells. Third, we explored if the immune-suppressive activity of MM-LDN could be recovered by treatment *in-vitro* with 200 nM epacadostat, an IDO-1 inhibitor currently under investigation in phase I-II trials of immunotherapy in solid cancers. Fourth, we explored if tryptophan shortage could induce an adaptive response to MM cells MM1.s, OPM2 and U266 *in vitro* and mediate refractoriness to bortezomib, melphalan and lenalidomide.
Results: IDO-1 was increased in both bone marrow and peripheral blood of MM patients compared to MGUS and healthy subjects (p=0.002). Conversely, tryptophan was reduced (more in peripheral blood than in bone marrow) in MM *versus* MGUS patients and kynurenine (a product of tryptophan degradation) increased (p=0.001). T- cell function, evaluated as expression of HLA-DR and CFSE expression upon stimulation with 5ng/mL phytohaemagglutinin (PHA) for 72 hours, was hampered by co-colture at ratio 1:4 with MM-derived HDN, and only partially reverted by treatment with epacadostat.
MM cells expressed IDO-1 but their viability was not affected by exposure to epacadostat up to 72 hours. Tryptophan shortage (1000-10nM) did not affect cell proliferation and cell cycle of MM cell lines tested either, while induced T-cell apoptosis within 48 hours. In two human myeloma cell lines MM1.s and U266, progressive tryptophan shortage induced an adaptive response through increased expression, time and dose-dependent, of ATF4-ASNS-CHOP-GADD34, part of GCN2 signaling, followed by autophagy induction and fitness marker IRF4 and Blimp1 upon 96 hours of starvation. *In-vitro*, sub-toxic treatment with 5nM bortezomib, 10uM lenalidomide or 10uM melphalan for 24 hours showed synergic effect only between melphalan and epacadostat.
Summary and Conclusions: IDO-1 and tryptophan shortage are associated to MM progression. HDN are IDO-1 positive and mediate immune-suppression that can be reverted only partially by treatment with 200nM epacadostat. Epacadostat has synergic effect with melphalan *in vitro*. Experiments *in vivo* are ongoing to understand the contribute of IDO-1 in MM establishment in immune competent models of disease.

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CHARACTERIZATION OF ST3GAL6-AS1, A NOVEL LNCRNA DEREGULATED IN MULTIPLE MYELOMA

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Background: During the last two decades, long non-coding RNAs (lncRNAs) relevance has been highlighted by a large number of studies. LncRNAs are non-protein-coding transcripts, longer than 200 nucleotides, which can act to regulate gene expression and be involved in cancer. More recently, some groups evidenced the impact of deregulated lncRNAs in Multiple Myeloma (MM). In previous report, we demonstrated that deregulated patterns of lncRNAs expression are associated with distinct MM molecular subtypes (Ronchetti *et al.*, Oncotarget, 2016).
Aims: (i) Confirming and extending data on lncRNA expression in MM (ii) Characterizing the RNA structure and explore the functional role in MM human cell lines (HMCLs) of ST3GAL6-AS1, the unique up-modulated lncRNA in MM patients compared with healthy donors.
Methods: A custom annotation pipeline was used to investigate lncRNA profiles on GeneChip® Human Gene 2.0 ST microarray in highly purified bone marrow PCs from 50 MM primary tumors and 4 normal donors. Real-time PCR (QT-PCR) was performed to confirm array data. Predicted splicing of ST3GAL6-AS1 transcripts were examined by qualitative PCR and Sanger sequencing in HMCLs and primary tumors. lncRNAs subcellular localization was evaluated by fractionated HMCLs nuclear and cytoplasmic RNA. HMCLs were treated with actinomycin D to determine the half-life of the lncRNAs. siRNA silencing of ST3GAL6 on HMCLs was performed by Neon Transfection system.
Results: We analyzed the long non-coding RNA fraction of the transcriptome of 50 MM patients using arrays that investigate more than unique 10000 sequences. First, we confirmed that the expression of the 5% most variable lncRNAs across the dataset grouped MM samples based on the main cytogenetics prognostic alterations (p<0.0001). One-hundred sixty-four lncRNAs showed significant differential expression in hyperdiploid, 11q14, 4p16 and MAF translocations groups. Importantly, we found ST3GAL6-AS1 as the unique significant overexpress lncRNA in MM samples compared to healthy donors. ST3GAL6-AS1 maps to 3q12.1 and is antisense to ST3GAL6, a protein involved in homing and *in vivo* engraftment of HMCLs and correlated with shorter overall survival in MM patients (Glavey *et al.*, Blood, 2014). The validation of array data by QT-PCR confirmed the overexpression of ST3GAL6-AS1 in MM sample. Analysis on HMCLs revealed that ST3GAL6-AS1 was overexpressed and equally localised in nuclear and cytoplasmic fractions. Furthermore, molecular analysis of the lncRNA in HMCLs and primary tumors showed the presence of a polymorphic splicing nucleotide variant (rs13065271) with the retention of a 128bp intron in the transcript. In homozygous mutated HMCLs we observed a prevalent nuclear localization of ST3GAL6-AS1, as well as a lower expression and reduced half-life of its transcripts. Moreover, ST3GAL6-AS1 and ST3GAL6 displayed a significant correlation in their expression levels. siRNA silencing of ST3GAL6 in HMCLs caused down-regulation of ST3GAL6-AS1, suggesting a possible co-regulation mechanism.
Summary and Conclusions: Our data indicate that ST3GAL6-AS1 is significantly deregulated in MM patients. Furthermore, the occurrence of a polymorphic variant leading to an alternative splicing in ST3GAL6-AS1 may have potential relevance in the transcript stability and its functional role.

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MULTIPLE MYELOMA RELATED ANGIOGENESIS: ROLE OF NOTCH-JAG AXIS IN MODULATING ENDOTHELIAL CELLS BEHAVIOR

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Background: Multiple myeloma (MM) is an incurable malignancy characterized by plasma cells accumulation in the bone marrow (BM). MM cells are able to shape the BM niche, inducing BM resident cells to support malignant cells survival and proliferation. In this process, a key role is played by alterations of Notch pathway. Indeed, the overexpression of Notch1/2 receptors and Jag1/2 ligands by MM cells promotes Notch hyperactivation not only in tumor cells, but also in the surrounding normal cells, stimulating different processes aimed to sustain MM cell progression. One of the mechanisms that promotes MM growth and contribute to its fatal outcome is the increased level of angiogenesis within BM. Interestingly, even if the link between MM progression and the angiogenic switch is well known, the contribution of Notch activity in this process still needs to be clarified.
Aims: This study aims to investigate the role of Notch-Jag axis in MM-associated angiogenesis evaluating the contribution of MM cells and of BMSCs.
Methods: We used 3 different cell lines, RPMI8226, U266 and OPM2 in which the Notch ligands, Jag1/2, were silenced ($MM^{KDJAG1/2}$) using specific siRNAs to study the role of Notch-Jag axis in promoting angiogenesis. The human pulmonary artery endothelial cells (HPAECs) were used to mimic the endothelial compartment while BMSCs were represented by the GFP^{+}HS5 cell line. The outcome of Jag1/2 silencing on MM-ECs interaction or on MM cells ability to secrete pro-angiogenic factors was analyzed using three assays that allowed us to evaluate different biological processes involved in tumor angiogenesis: motility (wound healing assays), adhesion on ECM-like substrate (adhesion assay) and EC tube formation assay. Finally, we analyzed MM capability to activate Notch signaling in BMSCs and induce the secretion of VEGF by qRT-PCR and flow cytometry. To investi-

gate the biological outcome of these variations, the above-mentioned assays were performed using CM collected from MM-BMSCs co-culture system.
Results: Our results indicate that MM cells promote tube formation both through direct contact and by releasing soluble factors. This effect is significantly reduced in the absence of Jag1/2 ligands, sustaining the hypothesis of a key role of Notch signaling in ECs stimulation. Comparable results were obtained in wound healing and in adhesion assays. Experiments performed on MM-BMSCs co-culture system showed that MM cells are able to boost BMSCs ability to produce the angiogenic factor VEGF. Interestingly, this effect is reverted by Jag1/2 silencing. As expected, MM-driven modulation of stromal-derived VEGF impacts on EC motility, adhesion and tube formation.
Summary and Conclusions: These findings indicate a novel role for the Notch-Jag axis in MM ability to promote angiogenesis in the BM microenvironment. Tumor-associated angiogenesis is supported by MM-ECs direct interaction and by the action of Notch-dependent MM-derived soluble factors. Moreover, Notch-Jag axis is involved in promoting stromal-derived VEGF production that further stimulates angiogenesis.

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ADIPORON, THE FIRST ORAL ADIPONECTIN RECEPTOR AGONIST, INHIBITS THE PROLIFERATION OF MYELOMA CELLS VIA THE AMPK / MTOR /AUTOPHAGY SIGNALING PATHWAY

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Background: Multiple myeloma (MM) is the second most common hematological malignancies. New agent therapy and hematopoietic stem cell transplantation have significantly improved the survival of MM, but patients will eventually progress to death. To develop new drugs with different mechanisms of action is the fundamental ways to overcome this problem. Adiponectin has been reported to play an important role in the pathogenesis of MM and is a potential therapeutic target. However, it's difficult to directly use adiponectin in clinical practice. AdipoRon, the first oral adiponectin receptor agonist, plays a similar role as adiponectin in improving glycolipid metabolism while the role of AdipoRon in hematological malignancies remains blank.
Aims: The aim of this study was to investigate the inhibitory effects of AdipoRon on proliferation of myeloma cells and to explore the underlying mechanisms.
Methods: RT-PCR was used to quantify the AdipoR1 and AdipoR2 mRNA copy number in the bone marrow cells from 21 patients with MM. 23 normal marrow samples were served as control. Informed consent was obtained for every marrow sample. The cell proliferation was detected by CCK-8 and cell apoptosis was analyzed by flow cytometry. Western blot was used to determine the protein level of the signaling pathway.
Results: We found that the expression of AdipoR1 in MM was significantly higher than that in normal controls, while the expression of AdipoR2 in MM was significantly lower than that in normal controls, suggesting that adiponectin receptors are differentially expressed between MM patients and normal individuals. AdipoRon significantly inhibited the proliferation of MM cell lines Sp2 and MPC-11 in a concentration-dependent and time-dependent manner without affecting the proliferation of normal mesenchymal stem cell. Flow cytometry showed that AdipoRon induced apoptosis of MPC-11 cells in a concentration-dependent manner; Western blot showed that AdipoRon increased the expression of apoptosis-related proteins cleaved caspase-3 and cleaved PARP. AdipoRon upregulated p-AMPK and its downstream p-ACC and down-regulated p-mTOR in MPC-11. In addition, AdipoRon upregulated LC3- II / LC3-I level and down-regulated the protein level of p62, suggesting the activation of autophagy.
Summary and Conclusions: AdipoRon, the first adiponectin receptor agonist, could inhibit the proliferation and induce the apoptosis of myeloma cells, activate AMPK, inhibit the mTOR signaling pathway and activate autophagy in myeloma cells, suggesting that AdipoRon might play an important role in anti-proliferation of myeloma cells via the AMPK/mTOR/autophagy signaling pathway. The results need to be further verified *in vivo*.

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CLINICAL RELEVANCE OF LONG NON CODING RNA IN MULTIPLE MYELOMA: RETROSPECTIVE MONOCENTRIC STUDY

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Background: Multiple myeloma (MM) is a malignant proliferation of bone marrow plasma cells (BMPC) characterized by highly heterogeneous genetic background and clinical course. Long non-coding (lnc)-RNA represents the largest class of non-protein coding genes in the human genome. LncRNAs deregulation has been reported to promote tumor formation, progression and metastasis in many types of cancer. Their role in MM is progressively expanding. In previous report, specific lncRNA transcriptional profile has been described in subgroups characterized by molecular/cytogenetics alteration or progressive stages of the disease (Ronchetti *et al.*, 2016).
Aims: This study was aimed at evaluating the correlation between lncRNA expression profiles and clinical variables in MM at diagnosis.
Methods: We retrospectively collected the clinical and laboratory data of 80 MM patients (pts), who have been admitted to our Hematology unit from 2000 to 2014. Complete clinical data were available together with FISH/cytogenetics information and transcriptional profiles generated on GeneChip® Human Gene 1.0st arrays.
Results: The median age at diagnosis was 67 years, 38% of pts had anemia, 11% renal failure, only 4% was hypercalcemic, and 57% showed bone damage. BMPC infiltrate was greater than 60% in half of the cases, while extramedullary localizations occurred in only 2 pts. Del(13) was present in 51% of pts; del(17) in 10.1%; t(4; 14) translocation in 10%; t(11;14) in 25%; t(14;16) or t(14;20) in 7.5%; t(6;14) in 6.3%; 36.6% pts were hyperdiploid, 52.7% had 1q gain and 19% had 1p loss. Fit pts underwent autologous stem cells transplantation (ASCT), whereas others were treated with chemiotherapy, immunomodulatory drugs or Bortezomib. The median overall survival (OS) was 77 months and the time to next treatment (TNT) was 22 months. According to ISS), pts in stage I were 25, in stage II 30, in stage III 23. According to R-ISS, pts in stage I were 14, in stage II 52, and in stage III 12. R-ISS increased the number of patients in category II and effectively stratified pts in term of OS e TNT (p=0.005 and p=0.0003, respectively). We evaluated the association between lncRNA expression, assessed as a continuous variable, and outcome using *globaltest* package in R software, and identified 12 and 1 lncRNAs whose expression showed respectively negative and positive association with OS. Considering TNT, 15 lncRNAs were positively and 1 negatively correlated. In particular, the most significant correlation with poor outcome, in terms of both time to therapy and survival, was found for LIN00599, a lncRNA that was described to regulate the transcription through endogenous competition with microRNAs (specifically miR-4306, miR-185-5p, miR-4644). No correlation was found between lncRNA expression and response to therapy, independently of the type of treatment and the number of previous therapies. Additionally, our data indicated absence of relationship between lncRNA expression and ASCT therapy. Finally, the expression of each of the 13 lncRNAs associated with OS and the 16 associated with TNT was then tested as dichotomic variable in association with ISS and R-ISS. Our analysis revealed that none of the tested lncRNA improved ISS models.
Summary and Conclusions: In our retrospective cohort, representative of the main molecular alterations in MM, we unraveled lncRNA transcripts whose expression could be associated with outcome. However, lncRNA expression did not improve R-ISS staging model, which in our cohort retained the highest prognostic value.

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DRUG SENSITIVITY SCREENING IN MULTIPLE MYELOMA (MM) FOR PRECISION CANCER THERAPY

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Background: Chronic Lymphocytic Leukemia (CLL) and Multiple Myeloma (MM) are considered incurable. Although modern treatment regimens prolong survival, CLL and MM eventually relapse. Current challenges include design of optimal treatment for individual patients based on characterization of the tumor and its intratumor heterogeneity as observed by whole genome sequencing. Efficient therapies require a personalized approach that combines targeting malignant plasma cells as well as the support malignant plasma cells receive from the tumor microenvironment. Another major limitation is that there exists no efficient approach to identify the most efficient drugs for each patient and also for different cancer stage. Using our drug sensitivity screening platform, we aim to address the limitation in identifying the efficient drugs for individual patients.