Session 17: Tumor Microenvironment in Cancer Progression

Lectures

L17.1

Tumor suppressor function of FOXO1 in diffuse large B-cell lymphomas: mechanisms of regulation and rational targeting strategies

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Introduction: In normal B lymphocytes, B-cell receptor (BCR)-induced activation of PI3K-AKT kinases and subsequent inactivation of FOXO1 is a critical pro-survival component of tonic BCR signaling. In murine models, conditional deletion of FOXO1 protected quiescent peripheral B cells from apoptosis mediated by inducible loss of the BCR, demonstrating that PI3K-AKT-FOXO1 axis plays a central role in B-cell homeostasis. Disruption of the BCR signaling by SYK inhibitor leads also to the apoptosis of BCR-dependent DLBCLs, at least in part via a mechanism involving decreased activity of PI3K/AKT axis. We investigated the role of FOXO1 in the toxicity of BCR pathway/SYK inhibition in human BCR-dependent lymphomas.
Methods: BCR-dependent DLBCL cell lines were incubated with SYK inhibitor and AKT phosphorylation, FOXO1 activation and transcriptional activity were assessed by phospho-specific flow cytometry, Western Blot and qPCR. FOXO1 knock-down in DLBCL cell was achieved with shRNA. The expression of p-SYK and FOXO1 in DLBCL samples was assessed by immunohistochemistry.
Results: Since FOXO1 is a major effector of tonic BCR signaling, we assessed the activity of FOXO1 in DLBCL cells after SYK inhibition. In all tested cell lines, AKT and FOXO1 phosphorylations decreased after incubation with a SYK inhibitor. Diminished FOXO1 phosphorylation resulted in its nuclear relocalization and induction of FOXO1-dependent gene expression. Constitutively nuclear and transcriptionally active FOXO1-3A mutant induced G1/S cell cycle arrest and triggered apoptosis, whereas wild-type FOXO1 did not change proliferation or cellular viability, demonstrating that FOXO1 activation is sufficient to induce apoptosis of DLBCL cells. Cells with depleted FOXO1 exhibited lower sensitivity to SYK inhibitor than control cells. We further characterized the mechanisms of FOXO1 – induced cell death, highlighting the role of increased expression of a proapoptotic BCL2-family member, HRK. Finally, we demonstrate that FOXO1 activity in DLBCL is regulated by redox-dependent acetylation by p300 acetyltransferase.
Conclusions: Taken together, these results demonstrate the role AKT and FOXO1 as mediators of proapoptotic activity of BCR/SYK blockade in DLBCLs. Since functional FOXO1 is required for proapoptotic activity of SYK inhibitor, these results highlight a potential role of FOXO1 in identifying patients unlikely to respond to this drug. We also describe a redox-dependent mechanism regulating FOXO1's proapoptotic signaling.

L17.2

Role of anti-inflammatory protein MCPIP1 in clear cell renal cell carcinoma progression

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Clear cell renal cell carcinoma (ccRCC) is the most common type of renal cell carcinomas, which forms tumours strongly supplied with blood vessels. At the time of diagnosis, metastasis is found in one third of patients. Despite targeted therapy, the average survival time for patients with metastatic renal cancer varies between three months and two years.
Inflammatory response is a crucial component of the tumour development, and it has been shown that pro-inflammatory cytokines, IL-1 or IL-6 may be involved in angiogenesis and tumour growth, as well as in invasion and metastasis.
Monocyte chemotactic protein 1-induced protein 1 (MCPIP1) acts mainly as an endonuclease that degrades the mRNA of proinflammatory cytokines, such as IL-6, IL-1, IL-12 and IL-2. MCPIP1 regulates NFκB and AP1 activity and may suppress miRNA biosynthesis. Last findings shows that MCPIP1 also regulates viability and proliferation of tumour cells, degrades the mRNA of antiapoptotic gene transcripts in breast cancer cells and reduces tumour growth and metastatic disease of breast cancer *in vivo*.
Our data indicates that MCPIP1 protein level, varied depending on the tumour grade and significantly decrease during ccRCC progression. We have already demonstrated that MCPIP1 is responsible for better tumour vascularity and the secretion of proangiogenic factors. MCPIP1 protein regulates the expression of SDF-1 and VEGF in ccRCC *in vitro* and *in vivo*. Moreover, tumour cells with MCPIP1 downregulation affect endothelial cells motility and formation of tubular-like structures. Our study revealed that MCPIP1 downregulation promotes the acquisition of mesenchymal phenotype and metastatic spread of ccRCC cells *in vitro* and *in vivo*. The silencing of MCPIP1 suppressed E-cadherin and upregulated β-catenin and vimentin and induced the expression of E-cadherin repressors, Snail and ZEB2. Our study showed that MCPIP1 level decreases during ccRCC progression together with MET receptor upregulation. Increased phosphorylation of MET receptor may play significant role in the acquisition of resistance to RTKs inhibitors in ccRCC.
Our findings shows, that MCPIP1 overexpression in ccRCC cells impairs the malignant phenotype of ccRCC cells.
Obtained results may contribute to increased understanding of the biology of clear cell renal cell carcinoma, which in the future may help in identifying new, more effective therapeutic purposes or improving existing ones.

L17.3

Tumour microenvironment in luminal breast cancer progression

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Stromal stimuli mediated by growth factor receptors is involved in development of breast cancer (BCa) resistance to anti-ER (Estrogen Receptor) treatment. Mutations of FGFR2 (Fibroblasts Growth Factor Receptor 2) were shown to be correlated with increased risk of breast cancer, although molecular mechanism of FGFR2 action for BCa development and progression was never fully elucidated. Our studies indicated that FGFR2 mediated cancer-associated fibroblasts-originating signaling which lead to activation of ER and PR (Progesterone Receptor). The process has been deeply studied and proved to contribute to progression of luminal breast cancer and development of resistance to routine anti-ER therapies.

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Oral presentations

O17.1

Chemotherapeutics-treated cancer cells display stem-like and senescent cell features

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Anticancer therapies, including chemotherapy, tend to trigger therapy-induced senescence (TIS) in cancer cells, that is linked to irreversible growth arrest. Although, accumulation of senescent cancer cells was reported to decrease survival of patients after chemotherapy. Therefore, we studied whether TIS escape may follow treatment with chemotherapeutics used clinically: 5-fluorouracil (5-FU), oxaliplatin (OXA) and irinotecan (IRINO). The colon cancer cells treated with 5-FU or IRINO, but not with OXA, exhibited several hallmarks of TIS. At the same time, we identified a subpopulation of senescent colon cancer cells with features of stemness. Furthermore, rare, polyploid cells exhibited blastocyst-like morphology and produced progeny. Our study shows that a subpopulation of chemotherapeutics-treated colon cancer cells display a specific phenotype being a combination of stem-like and senescent cell features. This may contribute to their dormancy, resistance to chemotherapy and ability to re-grow cancer after completion of therapeutic intervention.

Acknowledgements:
This work was supported by the Foundation for Polish Science co-financed by the European Union under the European Social Fund (grant 125/UD/SKILLS/2013), the Ministry of Science and Higher Education (grant IP2012 062172), and the National Science Center (grants 2013/09/B/NZ3/01389 and 2012/05/E/ST2/02180).

O17.2

Preoperative high platelets counts and low hemoglobin correlate to mesenchymal phenotype of circulating tumor cells and worse clinical outcome in human breast carcinoma

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Peripheral blood might serve as a source of cells and molecules potentially utile in cancer diagnostics, and a playground for interactions of tumor cells being in transit with blood cells and other circulating factors (e.g. cytokines). Thus, clinical relevance of blood-derived factors has to be evaluated in context of patients' outcome, mechanisms underlying tumor dissemination or influencing the efficiency of metastatic process.
In the current study the relationship of circulating tumor cells (CTCs), their phenotype, blood count and cytokines to breast cancer progression was tested (n=75). The adverse impact of CTCs on patients' overall survival (OS) was described before. Here, the high platelets count (↑PC) and the low hemoglobin concentration (↓Hb) correlated to the mesenchymal CTCs (p=0.039 and p=0.034, respectively) and shorter OS (p=0.009 and p=0.001, respectively). ↑PC was associated with the high content of intratumoral stroma (p=0.050) and the increased concentration of cytokines related to platelets activation or production in bone marrow (i.e. APRIL, ENA78, HGF, IL9, IP10, MDC, MMP1 and SCF), whereas ↓Hb to the high concentration of ENA78 and the low concentration of PDGF-BB (n=36, all p<0.05).
In conclusion, the assessment of CTC, platelets and Hb have prognostic potential in breast carcinoma and might reveal the complex network of environmental elements supporting tumor progression potentially *via* induction of primary tumor–blood–bone marrow–blood axis.

O17.3

The influence of metabolism on the phenotype of astrocytoma U-251 MG cell line

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Metabolism of highly proliferative normal cells and cancer cells, as opposed to normal, differentiated cells, is based on glycolysis. The process doesn't depend on oxygen availability and leads to lactate production. This phenomenon is called aerobic glycolysis or "Warburg effect". Cells excrete lactate what causes extracellular environment acidification. This kind of metabolism can be beneficial for cancer cells for many reasons, including rapid biomass generation, efficient intake of glucose and glutamine, impaired immune response and activation of metalloproteinases. Nowadays, many anti-cancer therapies are directed against metabolic pathways.
The influence of metabolism, especially glycolysis, on the regulation of interleukin 6 (IL-6) and matrix metalloproteinase 2 (MMP-2) level in astrocytoma cells is the topic of this research. IL-6 is a pro-inflammatory cytokine that can contribute to cancer progression through its anti-apoptotic and pro-proliferative effect. MMPs are special enzymes, enabling cancer cells to degrade the extracellular matrix (ECM). Human astrocytoma U-251 MG cell line was used for the study. The level of glycolysis in this cell line is so high that neither LPS nor IL-1β can increase it further. After inhibition of glycolysis by 2-deoxyglucose (2-DG) we can observe changes on the IL-6 and MMP-2 mRNA and protein level. We also investigate promoter activation, mRNA stabilization and cell migration after 2-DG treatment.

O17.4

Extracellular vesicles secreted by colorectal cancer cell line HT29 overexpressing Snail can fuse with and activate the cells constituting metastatic niche

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Tumor cells extracellular vesicles (EVs), that include microvesicles (MV) and exosomes, has been considered messengers in intercellular communication, mediate the formation of metastatic niches and affect cancer progression. We have established the clones of human colorectal cancer HT29 cell line that stably express Snail (HT29-Snail), a key transcription factor of the epithelial-mesenchymal transition. Further, we isolated EVs from cell conditioned media using differential centrifugations and ultracentrifugation.
We confirmed the identity of exosomes and MV fractions of EVs by transmission electron microscopy. CD63 marker but not cytochrome *c*, was present on EVs as judged by Western blot, that confirms the purity of vesicles. The exosomes and MVs were labelled using PKH67 dye to examine their uptake into human endothelial cells (HUVEC) and monocyte/macrophage-like cell line THP-1. We also quantified the amounts of various cytokines secreted by HUVEC and THP-1 after the uptake of the EVs isolated from media from either control or HT29-Snail cells.
We found that EVs from HT29-Snail cells that were incorporated into the cells constituting pre-metastatic niche, significantly increased release of IL-8 a chemokine that has pro-angiogenic and pro-inflammatory properties. It confirms the role of Snail in these processes and provides inside into the mechanism by which Snail and EVs contribute to modification of pre-metastatic niches.

Acknowledgments:
Supported by the NCN project 2011/02/A/NZ3/00068.

O17.5

Tumor-associated neutral endopeptidase (tNEP) regulates cell proliferation and TGF-β production in cultures and co-cultures of colon cancer cells with colon fibroblasts

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Recently, it has been shown that tNEP promotes growth and invasiveness of colon cancer cells (CC) (1,2). However, there is no information whether tNEP is involved in cooperation between CC cells and colon fibroblasts (CFs; component of the tumor microenvironment) or influences the production of TGF-β (cytokine that promotes CC cell invasiveness) in the tumor microenvironment. Therefore, we examined cell proliferation (with the BrdU assay) in two human tNEP-expressing CC cell lines (LS180 and SW620) cultured in growth medium or co-cultured in transwells (0.4mM) with human CFs (CCD-18Co cell line) and/or CFs-conditioned medium. Additionally, CC cells with inhibited activity (by thiorphan) or silenced expression (by siRNA) of NEP were used in the experiments mentioned above. The ELISA method was used to assess the level of TGF-β in supernatants from cultures and co-cultures. The results showed that the co-culture of tNEP-expressing cells with the CFs and/or with the CFs-conditioned medium did not affect CC cell proliferation. In contrast, the tNEP inactivation or silencing inhibited CC cell proliferation in comparison with the control, and the co-culture with the CFs and/or the CFs-conditioned medium led to even greater inhibition of proliferation. The tNEP inactivation or silencing slightly decreased the level of TGF-β both in the culture and in the co-culture with the CFs and/or the CFs-conditioned medium. The results suggest that tNEP is at least partially involved in creation of the CC microenvironment.

Acknowledgments:
This work was supported by Grant No 2012/07/B/NZ3/00209 from NCN, Poland.

Posters

P17.1

Spontaneous senescence of ovarian cancer cells: first observations and further research directions

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According to a classic theory, cellular senescence limits the mitotic lifespan exclusively in normal somatic cells. Last decades have provided evidence that senescence may also be induced in cancer cells, in response to a chemotherapy. In addition, some reports suggest that senescence of cancer cells may also occur spontaneously.
Recently we observed that a significant fraction of ovarian cancer cells, either in tumors or in culture conditions, display signs [SA-b-Gal(+)/g-H2A.X(+)] of senescence. Significantly, patients from whom the cells were isolated had not received any chemotherapy, which implies that the senescence was a spontaneous process.
According to these findings we propose that spontaneous senescence of ovarian cancer cells may exert a pro-tumoral activity, similarly to various types of senescent somatic cells. This effect may proceed either in the autocrine manner (e.g. through the senescence-associated secretory phenotype; SASP) or in the paracrine fashion by the induction of certain pro-oncogenic features in the normal peritoneal cells. We also hypothesize that the outcome of spontaneous cancer cell senescence (pro-cancer) may be different from that of the drug-induced phenomenon (anti-cancer). The opposite effects may result from different mechanisms and phenotypes characterizing cells undergoing both types of senescence.

Acknowledgments:
The study was supported by the grant from the National Science Centre, Poland (# 2017/25/B/NZ3/00122).

P17.2

Carboplatin and paclitaxel induce premature senescence in normal peritoneal mesothelial cells and fibroblasts

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The gold standard in the treatment of ovarian cancer is carboplatin (CPT) combined with paclitaxel (PCT). The mechanism by which these drugs restrict the malignancy is well established. At the same time, very little is known about effects exerted by these drugs on biology of normal peritoneal cells. Here we examined whether CPT and PCT may induce senescence of primary, omental mesothelial cells (HPMCs) and fibroblasts (HPFBs), that is a process known to support the spread of ovarian cancer both *in vitro* and *in vivo*. The drugs were used at the doses at which they kill no more than 15% of cells (50 μM CPT + 25 nM PCT, and 25 μM CPT and 10 nM PCT for HPMCs and HPFBs, respectively). Experiments performed on 8 separate cultures of HPMCs and HPFBs revealed that cells subjected to the drugs display significantly increased expression of senescence-associated β-galactosidase, which coincided with the activation of DNA damage response (increased expression of histone γ-H2A.X and 53BP1). In addition, they displayed increased generation of reactive oxygen species. Significantly, effects exerted by the combination of the two drugs were uniformly stronger than those elicited by the drugs applied individually. Collectively, our results indicate that CPT and PCT may paradoxically promote ovarian cancer by inducing premature senescence of normal peritoneal cells.

Acknowledgements:
The study was supported by the grant from the National Science Centre, Poland (# 2017/25/B/NZ3/00122).

P17.3

Snail regulation of microRNAs during epithelial-to-mesenchymal transition in HT29 colorectal cancer cells

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Epithelial-to-mesenchymal transition (EMT) and its intermediate states in cancer cells, represents early stages of metastasis and promising target in colorectal cancer (CRC) therapy. It is important to identify markers and key pathways induced by EMT but the process is complex and depends on the type of cancer cells as well as tumor microenvironment.

Here we studied the microRNAs expression in the CRC cell line HT29 stably overexpressing Snail (HT29-Snail), the early EMT transcription factor. Snail did not affect the global microRNAs production but significantly triggered changes in individual miRs levels. It directly repressed miR-192 and miR-194 expression but also indirectly induced the increase in miR-205, let-7i and SNORD13. These changes were correlated with the reported previously transcriptomic alterations in HT29-Snail cells. We have also investigated how Snail affects the content of extracellular vehicles (EVs) released from CRC cells, the key components of intercellular communication. The content of EVs mirrored the intracellular changes in microRNAs expression between HT29 and HT29-Snail cells.

The therapeutic interventions at early steps of metastasis might interfere with microRNAs levels. Thus complex mRNA/microRNA interactions as well as the crosstalk between cancer cells and their microenvironment needs to be taken into account since our data suggest that all these processes are significantly altered.

Ackmowledgements:
Supported by NCN 2011/02/A/NZ3/00068 and 2016/22/E/NZ3/00341.

P17.4

The role of FGFR2-regulated E3 ubiquitin ligases in progesterone receptor (PR) turnover in breast cancer cells

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Breast cancer (BCa) is the most frequent cancer affecting women worldwide. Despite advances in early detection and comprehensive treatments, approximately 30% of patients with early-stage BCa still experience recurrent disease. Among the most common two BCa luminal subtypes (ER+) accounting for 50-70% of all BCa cases, luminal B tumors show lower or no expression of PR and more aggressive behaviour compared to luminal A cases. Interestingly, the loss of PR is accompanied by emergence of resistance to anti-ER treatment. In our previous studies we showed that FGFR2-triggered signalling is responsible for hyperactivation of PR followed by its proteasomal degradation in luminal BCa cell lines. Hence, these results encouraged us to focus on E3 ubiquitin ligases family and their involvement in FGFR2-mediated regulation of PR. We tested whether inhibition/silencing of selected E3 ligases affected FGFR-regulated PR level. Interestingly, we observed that inhibition of MDM2 as well as silencing of NEDD4-1 ubiquitinases decreased PR level and FGF1 treatment additionally enhanced these effects. Based on these unanticipated results we performed further analyses to reveal the role of indicated ubiquitinases in the molecular mechanism of PR loss which may contribute to luminal breast cancer progression.

P17.5

FGFR2 involvement in regulation of autophagy

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Autophagy is a physiological process of large molecules and damaged organelles degradation. The role of autophagy in oncogenesis is complex. At the early stages of cancers, autophagy suppresses tumor growth, whereas in advanced disease autophagy promotes cancer cell survival and resistance to therapeutics. Several studies showed autophagy-regulated activities of tumor microenvironment. On the other hand, tumor-associated cells (fibroblasts, macrophages) can also promote the process. Fibroblast Growth Factor Receptor 2 (FGFR2) was proved to mediate interaction between tumor and its microenvironment in luminal (ER-positive) breast cancer.Autophagy is a physiological process of large molecules and damaged organelles degradation. The role of autophagy in oncogenesis is complex. At the early stages of cancers, autophagy suppresses tumor growth, whereas in advanced disease autophagy promotes cancer cell survival and resistance to therapeutics. Several studies showed autophagy-regulated activities of tumor microenvironment. On the other hand, tumor-associated cells (fibroblasts, macrophages) can also promote the process. Fibroblast Growth Factor Receptor 2 (FGFR2) was proved to mediate interaction between tumor and its microenvironment in luminal (ER-positive) breast cancer.

In this project we analyzed an impact of FGFR2 signaling on the induction and/or inhibition of autophagy, by Western blot analysis and Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit, in two luminal A breast cancer cell lines (T47D and MCF7). FGFR2-dependent expression/activation of autophagy regulators (i.e. LC3, p62, Beclin-1 and members of Atg family) was analyzed. We also evaluated an involvement of autophagy in FGFR2-promoted cell growth in the presence of tamoxifen (anti-ER drug) and chloroquine (autophagy inhibitor) in 3D growth assay. Combined therapy based on FGFR and autophagy inhibitors/modulators may have potential application in patients with developed resistance to anti-ER drugs.

P17.6

Impact of oxygen conditions on apoptosis and cell cycle of human ovarian cancer cell line A2780 treated with cisplatin and resveratrol

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Epithelial ovarian cancer is one of the most malignant gynecological cancer in women. Hypoxia is an important microenvironmental factor shaping resistance to chemotherapeutic efficacy in tumors. Resveratrol, a natural compound, seems to be an attractive molecule for therapeutic purposes like cancer prevention. The aim of study was to examine the influence of oxygen tension on chemosensitivity of A2780 cell line. The cells were exposed to hypoxia (1% pO_2) or normoxia (~18% pO_2) and treated with resveratrol, cisplatin alone or in combination for 48h. In normoxia resveratrol was more pro-apoptotic than cisplatin and in combination the effect of drugs was synergistic. Hypoxia alone increased the percentage of apoptotic cells however it protected cells from death caused by resveratrol. This was not observed in the case of cisplatin. There was no effect of pO_2 on the cell cycle distribution after drug treatment; we noted accumulation of cells in G0/G1 phase and low rate of cells in G2/M phase in response to cisplatin and resveratrol both in normoxia and hypoxia. In conclusion, our observations suggest that hypoxia affects the pro-apoptotic potential of resveratrol and oxygen level needs to be taken into consideration when testing anticancer therapeutics.

Acknowledgements:
Study was supported by MIM intramural grant no 120/2011 and grant Kościuszko CRU/WIM/1435/2016 funded by Ministry of National Defence.

P17.7

Engineering and biosynthesis of TRF1 and TRF2 telomeric proteins for a novel anticancer chemotherapy development

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Telomeres are complex molecular structures present at the ends of eukaryotic chromosomes. Telomeric DNA is protected by a group of specific proteins that constitute the *shelterin* complex. We have designed, synthetized and cloned genes encoding two proteins from the complex: TRF1 and TRF2. The genes were optimized for expression in *Escherichia coli*. TRF1 interacts specifically with the duplex DNA and is implicated in telomere replication, telomere protection and telomere length maintenance. TRF2 is often described as the TRF1 paralog. TRF1 and TFR2 display significant structure similarity. Both proteins have three functional domains: the acidic TRF1 domain / the alkaline TRF2 domain, the central homodimerization domain (TRFH) and DNA binding domain (Myb).

We designed, cloned and expressed six recombinant gene variants coding for TRF1/2, TRFH1/2 and Myb1/2. The proteins were successfully biosynthesized in *E. coli* expression system. Simple and efficient protein purification protocols were established. The obtained recombinant proteins will be used in screening tests of multiple potential chemical inhibitors of the functional *shelterin* complex formation and telomerase activity.