ROLE OF PHOSPHOPROTEINS INVOLVED IN CHEMORESISTANCE OF COLORECTAL CANCER STEM CELLS AND IMMUNOPHENOTYPIC COMPARATIVE ANALYSIS

Giorgio Stassi (a), Vincenzo Canzonieri (b)
(a) Dipartimento di Discipline Chirurgiche e Oncologiche, Università di Palermo, Italy
(b) National Cancer Institute (CRO), Aviano, Italy

Operative Unit held by Prof. Giorgio Stassi

Recent studies demonstrated that colon cancers contain a cellular subpopulation, with stem cell-like proprieties, able to initiate and sustain tumour growth. These cells, so-called "Cancer Initiating Cells" (CICs), express the transmembrane antigen CD133.

CD133 positive cells show slow proliferation rate, high expression of ABC (ATP-binding cassette) transporters and anti-apoptotic factors making them resistant to conventional therapies.

Preliminary phosphoproteomic data, have shown that the target proteins of survival pathway PI3K/Akt/mTOR express an altered phosphorylation profile in colon CICs compared to the normal counterpart, contributing to therapeutic resistance. Therefore, the main purpose of this project was to validate, by immunocito/histochemical analysis, the results previously obtained, in order to develop new therapeutic strategies selectively directed against the CICs. The analysis was conducted both on *ex vivo* tissue tumor, primary cells directly obtained from the tissue digest, freshly purified $CD133^+$, $CD133^-$ cells, spheres and differentiated cells from spheres.

$CD133^+$ and $CD133^-$ cells were purified directly from tumor samples, after dissociation by mechanical and enzymatic methods, by MACS (magnetic cell sorting) technology, using super-paramagnetic beads conjugated with a monoclonal antibody anti-CD133/1 and immediately propagated in specific stem cell medium. The differentiation was obtained from spheres cells after growth factors removal and addition of serum.

We found an altered phosphorylation profile, in particular in the proteins containing a consensus sequence directly phosphorylated by AKT, including the pro-apoptotic factor BAD, protein IKK, which may indirectly increase the activity of nuclear factor NF -kB and stimulate the trascription of anti-apoptotic genes, inhibitors of cyclin-kinase $p21^{waf1/CIP1}$ and $p27^{KIP1}$ and the serine/threonine kinase mTOR. Moreover, we observed that $CD133^+$ cells treated with chemotherapeutic agents, such as 5-fluorouracil and oxaliplatin, express high levels of proteins involved in cell cycle progression such as Survivin and Aurora A.

Finally, we observed that genotoxic damage, induced by chemotherapy agents, increases the Sam68 level expression. Sam68 is a protein that can bind to mRNA of CD44 protein regulating CD44 variants splicing. CD44 is a membrane glycoprotein involved in many cellular processes such as proliferation, adhesion and cell migration.

Our data confirm the results previously obtained by phospho-proteomic analysis and provide a promising basis to understand the resistance mechanisms to chemotherapy treatments of colon cancer.

Publications of the project

Cammareri P, Lombardo Y, Francipane MG, Bonventre S, Todaro M, Stassi G. Isolation and culture of colon cancer stem cells. *Methods Cell Biol* 2008;86:311-24.

Francipane MG, Alea MP, Lombardo Y, Todaro M, Medema JP, Stassi G. Crucial role of interleukin-4 in the survival of colon cancer stem cells. *Cancer Res* 2008;68(11):4022-5.

Todaro M, Lombardo Y, Francipane MG, Perez Alea M, Cammareri P, Iovino F, Di Stefano AB, Di Bernardo C, Agrusa A, Condorelli G, Walczak H, Stassi G. Apoptosis resistance in epithelial tumors is mediated by tumor-cell-derived interleukin-4. *Cell Death Differ* 2008;15(4):762-72. Epub 2008 Jan 18.

Todaro M, Perez Alea M, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, Tripodo C, Russ A, Gulotta G, Medema JP, Stassi G. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell* 2007;1(4):389-402.

Todaro M, Perez Alea M, Scopelliti A, Medema JP, Stassi G. IL-4- mediated drug resistance in colon cancer stem cells. *Cell Cycle* 2008;7(3):309-13. Epub 2007 Nov 30.

Vermeulen L, Sprick MR, Kemper K, Stassi G, Medema JP. Cancer stem cells – old concepts, new insights. *Cell Death Differ* 2008;15(6):947-58. Epub 2008 Feb 15.

Vermeulen L, Todaro M, de Sousa Mello F, Sprick MR, Kemper K, Perez Alea M, Richel DJ, Stassi G, Medema JP. Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. *Proc Natl Acad Sci USA* 2008;105(36):13427-32.

Patents

Hoeger T, Gieffers C, Stassi G, Todaro M, inventors; Antibody specific for Human IL-4 for Treatment of Cancer. Publ. n.: WO2007107349. August 2004.

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In this early stage we are observing 30 cases of colon adenocarcinoma classified by TMN method as T2N0M0 extracted from a case record of 800.

We have already started to search signals of CD133 positivity by immunohistochemistry, and by molecular biology methods we are observing expressions of PROM1 gene related to CD133. We expect to extend this analysis to other cases presenting with different stage classification.

One of the aims of this kind of studies is to improve the prognostic accuracy of TNM system in malignant colon tumours especially in those in non advanced stages.

Considering the fact that all tumours have a genetic basis, these studies are mainly aimed at identifying genetic and molecular markers having a possible prognostic and predictive significance.

Therefore we set forth the evaluation of transcriptional data of genes such as CCNE1, TEK, PROM 1 (CD133) and PROX1.

In normal tissues Cyclin E/cdk2 complex is directly phosphorylated, even in early stages while Non Phosphorylated Cyclin E/cdk2 complex is found in neoplastic tissues causing instability, and, as a consequence, Cyclin E disruption (CCNE1gene), and this mechanism should be the cause of the production loss in its specific m-RNA, finding an under-expressed signal.

To support this hypothesis further, we have considered the observation of the TEK gene expression mapping on chromosome n. 9. The function of this gene is to produce proteins belonging to the tyrosine kinase series involved in cAMP fall system control. We believe that expressed/under expressed CCNE1 gene should be found as direct consequence, according to the TEK gene activity state itself, underlining the early loss of control of the replication cell cycle.

PROM1 gene (CD133 protein) is peculiarly expressed in stem cells. Its expression will be correlated to the data provided by the above described investigations. We are confident that the use of LNA (locked nucleic acid) probe for *in situ* hybridization with micro-RNA 21 (miR-21) will provide a valid additional evaluation of the disease's biologic aggressiveness. miR-21 is in fact involved in early neoplastic onsets and is related with low survival rate. All additional prognostic information may therefore be evaluated together with the expression of PROM1. Finally PROX1, mapping on chromosome n 1, is an onco-suppressor that controls the early stages of cell differentiation process. It has been chosen to observe possible differences from normal expression in non-neoplastic cells and during the follow-up.

To sum up, the global evaluation will provide us with a valid means of early alterations analysis of those cases which, in later follow-up, had an unfavourable progress.

As regards rectal carcinomas we will evaluate the same biological machinery in a large case series (about 200 cases) taking also into account the end point of radio-chemotherapy effectiveness in terms of local dowstaging and residual disease assessment .

Publications of the project

Canzonieri V, Memeo L, Perin T, Colarossi C, Rossi D, De Maria R. Immunoistochemical expression of CD133 in a selected series of T2N0M0 colon cancers. Evaluation for prognostic purposes. Manuscript in preparation.

Schiappacassi M, Lovat F, Canzonieri V, Belletti B, Berton S, Di Stefano D, Vecchione A, Colombatti A, Baldassarre G. p27Kip1 expression inhibits glioblastoma growth, invasion, and tumor-induced neoangiogenesis. *Mol Cancer Ther* 2008;7(5):1164-75.