Genetics and Genomics: Identifying Disease Causing Genes

CELLULAR AND MOLECULAR MARKERS IN THE DEVELOPMENT AND PROGNOSIS OF LYMPHOPROLIFERATIVE DISORDERS

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Lymphoproliferative disorders are a set of malignant diseases characterized by abnormal proliferation of lymphocytes, also called B and T cells, which are derived from pluripotent hematopoietic stem cells in the bone marrow.

**LYMPHOPROLIFERATIVE DISORDERS**

- Lymphoproliferative disorders are a set of malignant diseases characterized by abnormal proliferation of lymphocytes, also called B and T cells, which are derived from pluripotent hematopoietic stem cells in the bone marrow.

**WHO, 2008**

- MM = a clonal plasma cells malignant disease
MM accounts for 1% of all malignancies and 10% of hematological malignancies.

MM is the second most frequent lymphoproliferative disorder.

Incidence:
- ~15,000 new cases/year in the USA
- No official information in Brazil
MULTIPLE MYELOMA

Chronic antigenic stimulation?

Genomic instability
- Translocations at 14q32 (50%)
- Deletion of chromosome 13 (50%)

PLASMACYTOPHOMA

Microenvironmental changes in bone marrow
- Increased angiogenesis
- Increased bone resorption

N-RAS, K-RAS (30%)
- p16 Methylation (40%)
- Secondary translocations?

Monoclonal gammopathy of undetermined significance

Infection?
- Inflammation?

Normal cell

Myeloma

Kyle & Rajkumar, 2004
MULTIPLE MYELOMA

New Diagnostic Criteria  International  Myeloma Working Group

• Plasma cells >=10% or plasmacytoma in biopsy and
• At least one end-organ disfunction (CRAB):
  ✓ HyperCalcemia
  ✓ Renal insufficiency
  ✓ Anemia
  ✓ Bone lesions

Rajkumar et al, IMWG, Lancet Oncology, 2014
CURRENT SITUATION

- Approval schemes with proteasome inhibitors (bortezomib, carfilzomib, ixazomib) and lenalidomide by the FDA

- MM = chronic, incurable disease

- The five-year survival rate is around 35 percent

- This situation highlights an urgent need for novel therapies for the treatment of multiple myeloma

Bone marrow aspirate, 1000X

Cho, CRI, 2015
CELLULAR AND MOLECULAR MARKERS IN THE DEVELOPMENT AND PROGNOSIS OF LYMPHOPROLIFERATIVE DISORDERS

Main objective of our group

Molecular and cellular biology techniques

Genes and proteins expression

in vitro functional studies in MM cell lines

in vivo studies using murine models for possible new drugs combination

Biomarkers

Clinical outcome

New therapeutical targets

FAPESP Clinical Cancer Genome Project initiative (2000-2008)
GENE EXPRESSION IN MULTIPLE MYELOMA: IDENTIFICATION OF TUMOR MARKERS AND POSSIBLE THERAPEUTIC TARGETS

1. Discipline of Hematology and Hemotherapy, Universidade Federal de São Paulo, UNIFESP, São Paulo, Brazil
2. Ludwig Institute for Cancer Research, São Paulo Branch, Brazil
3. Faculdade de Medicina de Ribeirão Preto/USP, Brazil
Presenting 5 Players in Multiple Myeloma

- Tumor proteins
- Dendritic cells
- T-cells
- BMSC (bone marrow stromal cells)
- Cancer stem cells

Feyler, Blood Reviews, 2013
NEW POSSIBLE TARGETS IN MM?

SAGE analysis highlights the importance of *p53csv, ddx5, mapkapk2* and *ranbp2* to multiple myeloma tumorigenesis☆

Roberta S. Felix¹, Gisele W.B. Colleoni¹, Otavia L. Caballero¹, Mihoko Yamamoto, Manuela S.S. Almeida, Valéria C.C. Andrada, Maria de Lourdes L.F. Chaffaiilhe, Wilson A. da Silva Jr., Maria Dripei
Begnami, Fernando Augusto Soares, Andrew J. Simpson, Marco Antonio Zago, André L. Vettore

¹ These authors contributed equally for this paper.

Adapted from Augen, Modern Drug Discovery, 2004
12 overexpressed genes in MM
(> 10 times than in normal plasma cells)

Real-time PCR validation of gene expression in 31 MM cases and 3 normal controls

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Function</th>
<th>Overexpression</th>
<th>Hypoexpression</th>
<th>Normoexpression</th>
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<tr>
<td>ZFHX1B</td>
<td>Zinc finger homeobox 1b</td>
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<td></td>
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<td>Cell metabolism</td>
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<td>XBP1*</td>
<td>X box binding protein 1</td>
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<td>PIM2*</td>
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<td>Cell metabolism</td>
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<td>Apoptosis</td>
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<td></td>
<td></td>
<td>Cell proliferation</td>
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<td>RANBP2*</td>
<td>Ran binding protein 2</td>
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<tr>
<td>TRIAP1/P53CSV</td>
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<td>Cell Cycle control</td>
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<td>67.8%</td>
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<td>DDX5*</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 5</td>
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<td>JUND*</td>
<td>Jun D proto-oncogene</td>
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<td>42%</td>
<td>0%</td>
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<td>Signaling</td>
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<td>MAPKAPK2*</td>
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<td>SP140 nuclear body protein</td>
<td>Immune response</td>
<td>67.8%</td>
<td>0%</td>
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<tr>
<td>NTN-1*</td>
<td>Nitrin 1</td>
<td>Cell metabolism</td>
<td>10%</td>
<td>0%</td>
<td>90%</td>
</tr>
</tbody>
</table>

Genes were considered differentially expressed in tumor samples when their expression levels showed at least a 4-fold increment or decrease in comparison to normal samples.

*MoAb available for immunohistochemistry

Felix et al, Cancer Letters, 2009
Guardian of cell cycle
TP53 Tumor suppressor gene

TRIAP1 - TP53 regulated inhibitor of apoptosis

TRIAP1 - Inhibits apoptosome formation and apoptosis

HSP70

http://www.biooncology.com/research-education/apoptosis/pathways
http://string-db.org/
Normal Cells

- Low level of genotoxic stress
- TRIAP1
- Hsp70
- Apoptosis inhibition
- Cell survival

Cancer Cells

- TRIAP1
- Hsp70
- Apoptosis inhibition
- Cell survival

TP53 REGULATED INHIBITOR OF APOPTOSIS 1 (TRIAP1) STABLE SILENCING INCREASES LATE APOPTOSIS BY UPREGULATION OF CASPASE 9 AND APAF1 IN RPMI8226 MULTIPLE MYELOMA CELL LINE

**TRIAP1 Expression**

![Bar chart showing TRIAP1 expression in different cell lines.](chart)

**Problem Validation in animal model**

![Graph showing % Apoptotic cells (annexinV+/PI+) in RPMI8226 cell line.](graph)

**Stable silencing**

![Graph showing TRIAP1 relative expression over time.](graph)

**No good antibody available for WB**

**U266 cell line: resistance to cell death after shRNA TRIAP1 transduction**

![Graph showing Caspase 9 activity in RPMI8226 cell line.](graph)

![Graph showing APAF1 relative expression in RPMI8226 cell line.](graph)

![Graph showing Caspase 3/7 activity in RPMI8226 cell line.](graph)

Fook-Alves et al., BBA Molecular Basis of Disease, 2016
U266 cell line has t(11;14), E419X missense mutation on RB1 gene, A161T missense mutation on TP53 gene and has shown deletions of chromosome 13 and 17p, which involve, respectively, RB1 and TP53 genes.

http://www.keatslab.org/myeloma-cell-lines/common-genetics

RPMI8226 cell line presents the t(14;16) translocation, TP53 point mutation [located in exon 7 (c.853G>A) resulting in a TP53 missense mutation (p.E285K)] and no Rb1 or TP53 deletion by FISH.

http://www.keatslab.org/myeloma-cell-lines/common-genetics

U266 = del 13 + del 17p

RPMI8226= no 13 or 17p deletions
HEAT SHOCK PROTEIN 70 (HSP70) AS THERAPEUTIC TARGET IN MULTIPLE MYELOMA: IN VITRO AND IN VIVO ANALYSES

AIMS:
✓ Development of a xenograft model of bioluminescent cell lines in immunodeficient mice.
✓ To explore the role of the inhibition of HSP70 (VER155008), with or without the proteasome inhibitor bortezomib, in vitro and in vivo.

Eugenio et al, 2016, unpublished data

http://www.springerimages.com/Images/Biomedicine/1-10.1007_s10585-007-9083-9-1

Eugenio et al, 2016, unpublished data
HEAT SHOCK PROTEIN 70 (HSP70) AS THERAPEUTIC TARGET IN MULTIPLE MYELOMA: IN VITRO APOPTOSIS ANALYSES

**RPMI8226**
- RPMI8226LUC WT
- RPMI8226LUC DMSO
- RPMI8226LUC + bort 100nM
- RPMI8226LUC + VER 155008 50μM
- RPMI8226LUC + VER 155008 80μM
- RPMI8226LUC comb 80μM
- RPMI8226LUC comb 50μM

**U266**
- U266LUC-PURO
- U266LUC-PURO DMSO
- U266LUC-PURO + VER155008 50μM
- U266LUC-PURO + VER155008 80μM
- U266LUC-PURO Comb 80μM
- U266LUC-PURO Comb 50μM
- U266LUC-PURO + bort 100nM

**Bortezomib - Proteasome inhibitor**
**VER 155008 - HSP70 inhibitor**

Eugenio et al, 2016, unpublished data

U266 Combo = >60% of late apoptosis induction after 48hs
HEAT SHOCK PROTEIN 70 (HSP70) AS THERAPEUTIC TARGET IN MULTIPLE MYELOMA: IN VIVO ANALYSES

RPMI8226-LUC-PURO cell line

VER15508

SALINE 0,9%

ROI = 4.703e+08 p/s

ROI = 4.223e+09 p/s

NOD.Cg-Prkdc<sup>scid</sup>I2rg<sup>tm1Wij</sup>/SzJ mice treated with VER155008 (40mg/kg) versus mice treated with saline sodium chloride injection after one week

Emission of bioluminescence of mice treated with bortezomib, VER155008 and combination versus untreated mice after one week of treatment

**A**

- treated
- saline 0,9%
- p<0.001

**B**

- treated
- saline 0,9%
- p>0.05

Next step - U266 mice model

**Eugenio et al, 2016, unpublished data**
Cancer/Testis Antigens (CTAs)

- Limited expression in somatic tissues (testis, fetal ovary, and placental cells)
- Regulation of CTAs expression by epigenetic mechanisms (methylatation)
- Since gonads are immune privileged organs, anti-CTA immune response could be tumor-specific

Other cancers:
- Breast cancer
- Melanoma
- “MAGE”
- Ovary Carcinoma
- Esophagus carcinoma

CTAs are attractive targets for immunotherapy in cancer

Adapted, Albert & Darnell, 2004
Prognostic impact of cancer/testis antigen expression in advanced stage multiple myeloma patients

Viviana C. C. Andrade, André L. Vattore, Roberta S. Felix, Manuela S. Almeida, Fabícola de Carvalho, José Salvador R. de Oliveira, Maria de Lourdes Lepas Farnani Chauffalla, Adalgisa Andriolo, Otavio L. Cavallaro, Marco Antonio Zago and Gisela W. B. Colombo

Keywords: human, multiple myeloma, CT antigens, mRNA, tissue distribution, prognosis

Number and percentage of MM patients with positive expression of the 13 CT antigens analyzed.

MAGE C1/CT7 – independent prognostic factor

Andrade et al, Cancer Immunity, 2008
What have we learned about MAGE-C1/CT7 in MM?

- Frequently expressed in MM (~70% cases)
- Nuclear and cytoplasmic expression – no plasmatic membrane expression
- Sometimes focal tumor expression
- Low spontaneous humoral response
- Homology with MAGE-C2/C10
- Independent prognostic factor in MM

Carvalho et al, Clinical and Developmental Immunology, 2012
Targeting MAGE-C1/CT7 Expression Increases Cell Sensitivity to the Proteasome Inhibitor Bortezomib in Multiple Myeloma Cell Lines

Fabrisio de Carvalho, Enico T. Costa, Ana Maria A. Camargo, Juliana C. Grego, Obel Masotti, Valeria C.C. Andrade, Bryan E. Strauss, Otavia I. Caballero, Djordje Ajanackovic, Giselle W.B. Colleoni

Published: November 16, 2011 • DOI: 10.1371/journal.pone.0027707

shRNA MAGEC1/CT7

shRNA MAGEC1/CT7 + bortezomib

Carvalho et al, Plos One, 2011
MAGEC1/CT7 has biological role in early stages of MM and may contribute to plasma-cell proliferation

Andrade et al, Cancer Immunity, 2008
Phase I/II clinical trial to investigate vaccination with CTA-loaded DCs in MM patients on LEN maintenance therapy

- VUB proposed to vaccinate MM patients on *LEN maintenance therapy with DCs matured* by electroporation with TriMix and co-electroporated with mRNA encoding the CTAs *MAGE-C1* and *MAGE-A3*
Ilustration – TriMixDC-MEL + Ipi

58y male
Stage IV-M1c
BRAFwt
Refractory to temozolomide

6 Jul 2011, Baseline
18 Oct 2011, +14 Weeks
16 Jan 2012, +27 Weeks

Courtesy Prof. Kris Thielemans, VUB
Tumor antigens

Dendritic cells

MSKCC - Autologous dendritic cell vaccine after autologous stem cell transplant
Nivolumab (anti-PD-1 antibody) and ipilimumab (anti-CTLA-4) antibody, are in a phase I trial testing in blood cancers, including multiple myeloma (NCT01592370)

Braga et al, Clinical and Developmental Immunology, 2014
IMMUNOPHETOYPIC AND FUNCTIONAL CHARACTERIZATION AND GENE EXPRESSION PROFILING OF MULTIPLE MYELOMA PATIENTS AND HEALTHY INDIVIDUALS BONE MARROW STROMAL CELLS

**MM Cases**

*N = 20*

- Newly diagnosed and untreated multiple myeloma patients

**Healthy Controls**

*N = 10*

- Healthy bone marrow donors for allogeneic transplantation

**Bone marrow aspiration**

**Bone marrow stromal cells isolation and expansion**

**Immunophenotypic characterization by flow cytometry**

**RNA extraction and gene expression profiling by microarray**

- GeneChip® Human Exon 1.0 ST Array (Affymetrix)
- 4 cases vs. 4 controls (both in triplicates)

**Bionformatics analysis and candidate genes selection**

**Protein Expression Validation**

**Early death in cell culture without malignant plasma cells support**

Fernando, 2016, unpublished data
Network analysis of differentially expressed genes in BMSCs – Cytoscape (version 3.3.0)

Enrichment analysis DAVID (version 6.7)

Fernando, 2016, unpublished data
MM-CSCs: controversy about the existence and cell phenotype

Boucher et al, 2012 - ALDH+/CD19+/CD34+/CD138-
Matsui et al, 2008 - ALDH+/CD20+/CD27+/CD34-/CD138-
Pilot study: FACS Aria analysis in MGUS case

FACS ARIA cell sorting

RNA extraction

RT² Profiler™ PCR Array Human Cancer Stem Cells (Qiagen, Hilden, Germany) - 84 genes related to CSCs

The genes are involved in various processes such as cell proliferation, self-renewal, asymmetric division, cell migration and signal transduction, among which are: NANOG, Oct4, SOX2, ALDH1A1, CD34 stemness-related genes

Possible MM-CSC

Fook-Alves and Dantonio, 2016, unpublished data
5 Players in Multiple Myeloma

- Tumor proteins/antigens
- Dendritic cells
- T-cells
- BMSC (bone marrow stromal cells)
- Cancer stem cells
Acknowledgements

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Adriana Feijó Evangelista

Adriana Franco Paes Leme

Adriana Bruscato Bortoluzo

Bryan Strauss
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