Integrated genomic analysis of microarrays of expression, genotyping and microRNAs in cells resistant to Methotrexate: Generation of Biological Association Networks

School of Pharmacy Research Seminars
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Cancer Therapy group
Department of Biochemistry and Molecular Biology
School of Pharmacy
University of Barcelona
Objective of the study: Anticancer Research based on Functional Genomics

Specific aims:

• To develop new anticancer treatments
  Identification of new targets
  - study their regulation at the molecular level

• To improve existing protocols
  – Combination with “old” drugs (chemosensitization)
  – Reduction of associated problems
    (e.g. drug resistance)
Model of the study:
Methotrexate therapy.

Glycine
Serine

\( \text{N}^5,\text{N}^{10}\text{-methylene-tetrahydrofolate} \)

\( \text{Glycine} \leftarrow \text{Serine} \)

\( \text{dUMP} \rightarrow \text{dTMP} \rightarrow \text{DNA} \)

\( \text{Dihydrofolate} \)

\( \text{NADPH} + \text{H}^+ \)

\( \text{DHFR} \)

\( \text{NADP}^+ \)

\( \text{MTX} \)
Development of cells resistant to MTX: a stepwise process.

- 10⁻⁸ M MTX
- Sub-confluency

- 30%
- 10⁻⁸ M MTX
- Sub-confluency

- 70%
- 3x10⁻⁸ M MTX
- Sub-confluency

- 30%
- 3x10⁻⁸ M MTX
- 70%
- 10⁻⁷ M MTX

Freezing of stocks...
Experimental approaches:

1) Gene expression profiles: using Affymetrix HG U133 A 2.0 Plus (54,700 probe sets)

2) Copy number determination: using qPCR and Affymetrix Genome-Wide Human SNP Array 6.0 (1.8x10^{-6} markers of genetic variation)

3) Network analysis (BANs) of gene expression results: using Pathway Architect (now within GeneSpring GX)

4) miRNA expression profiles: using Agilent Human miRNA Microarray Kit (V2) (800 miRNAs)
Experiment Design.

Sensitive cells

Cell Cultures

S1

S2

S3

Total RNA / Genomic DNA extraction

Labeling hybridization & scanning

S1

S2

S3

Fluorescence

Resistant cells

Cell Cultures

R1

R2

R3
Analysis of the results using GeneSpring GX

Silvia Peñuelas

Elisabet Selga
Clustering on conditions.
Microarray Affy HG U133A.2 plus (5-fold)
Over expressed 5-fold

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Fold Change</th>
<th>Description</th>
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<tbody>
<tr>
<td>201029_s_at</td>
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<td>CD99 antigen</td>
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<td>209699_x_at</td>
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<td>cytochrome P450, family 2, subfamily B, polypeptide 6</td>
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<td>214023_x_at</td>
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<td>tubulin, beta polypeptide paralog</td>
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<td>206561_s_at</td>
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<td>aldo-keto reductase family 1, member B10 (aldose reductase)</td>
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<td>40284_at</td>
<td>11.24</td>
<td>forhead box A2</td>
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<tr>
<td>211340_s_at</td>
<td>10.5</td>
<td>melanoma cell adhesion molecule</td>
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<td>202533_s_at</td>
<td>10.19</td>
<td>dihydrofolate reductase</td>
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<td>202834_at</td>
<td>8.703</td>
<td>angiotensinogen (serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antip</td>
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<td>203787_at</td>
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<td>jagged 1 (Alagille syndrome)</td>
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<td>6.144</td>
<td>protein kinase C, alpha</td>
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<td>6.133</td>
<td>collagen, type XVIII, alpha 1</td>
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<td>crystallin, lambda 1</td>
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<td>204489_s_at</td>
<td>5.637</td>
<td>CD44 antigen (homing function and Indian blood group system)</td>
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<td>201313_at</td>
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## AKRs expression in the microarray

<table>
<thead>
<tr>
<th>GenBank</th>
<th>Symbol</th>
<th>Gene name</th>
<th>Fold</th>
<th>Raw expression</th>
<th>p-value</th>
<th>Gene function</th>
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<tbody>
<tr>
<td>S68290</td>
<td>AKR1C1</td>
<td>Aldo-Keto reductase 1, member C1</td>
<td>19.3</td>
<td>206</td>
<td>3972</td>
<td>p &lt; 0.001 Xenobiotic metabolism</td>
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<tr>
<td>M33376</td>
<td>AKR1C2</td>
<td>Aldo-Keto reductase 1, member C2</td>
<td>21.3</td>
<td>154</td>
<td>3274</td>
<td>p &lt; 0.001 Bile acid transport</td>
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<tr>
<td>AB018580</td>
<td>AKR1C3</td>
<td>Aldo-Keto reductase 1, member C3</td>
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<td>NM_020299</td>
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<td>Aldo-Keto reductase 1, member B10</td>
<td>13.4</td>
<td>62.5</td>
<td>844</td>
<td>p &lt; 0.001 Aldheyde metabolism</td>
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</tbody>
</table>
Human AKR1C1 promoter sequence

Transcriptional regulation of AKR1C1 promoter and Effect of Sp1/Sp3 knockdown with siRNAs

HT29 sensitive cells

HT29 resistant cells

Treatment with siRNA against AKR1C1 increases MTX sensitivity in HT29 cells

Overexpression of AKR1C1 counteracts MTX S-phase arrest and apoptosis.

Chromosomal view of genes differentially expressed by 3 fold in MTX resistance

Selga et al. BMC Medical Genomics 2008, 1:35
<table>
<thead>
<tr>
<th>GenBank</th>
<th>Gene Name</th>
<th>Chromosome</th>
<th>Copy Number (Q-PCR)</th>
<th>Expression Microarrays</th>
<th>Validation (RT-PCR)</th>
<th>Gene Function</th>
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<tr>
<td>NM_002961</td>
<td>S100A4</td>
<td>1</td>
<td>0.85 ± 0.1</td>
<td>3.7 (p=5.5e^{-6})</td>
<td>5.68 ± 0.4</td>
<td>Angiogenesis</td>
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<tr>
<td>BU078629</td>
<td>ZFYVE16</td>
<td>5</td>
<td>16.81 ± 2.1</td>
<td>6.1 (p=7.7e^{-6})</td>
<td>6.7 ± 0.1</td>
<td>Zinc ion binding</td>
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<tr>
<td>AL144299</td>
<td>DHFR</td>
<td>5</td>
<td>16.09 ± 1.4</td>
<td>7.1 (p=1.2e^{-7})</td>
<td>11.05 ± 0.5</td>
<td>Nucleotide metabolism</td>
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<tr>
<td>NM_002439</td>
<td>MSH3</td>
<td>5</td>
<td>4.97 ± 0.5</td>
<td>3.9 (p=5.5e^{-6})</td>
<td>4.23 ± 0.4</td>
<td>Missmatch repair</td>
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<tr>
<td>AL912976</td>
<td>RASGRF2</td>
<td>5</td>
<td>17.76 ± 0.4</td>
<td>4.6 (p=8.9e^{-5})</td>
<td>6.10 ± 0.5</td>
<td>MAPK signaling</td>
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<tr>
<td>AF912976</td>
<td>SSBP2</td>
<td>5</td>
<td>10.27 ± 0.7</td>
<td>2.4 (P=3.4e^{-3})</td>
<td>2.96 ± 0.2</td>
<td>ss DNA binding</td>
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<tr>
<td>NM_022406</td>
<td>XRCC4</td>
<td>5</td>
<td>17.31 ± 1.1</td>
<td>7.1 (p=4.7e^{-6})</td>
<td>8.90 ± 2.3</td>
<td>ds break repair</td>
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<tr>
<td>U43328</td>
<td>HAPLN1</td>
<td>5</td>
<td>11.55 ± 0.1</td>
<td>147 (p=2.9e^{-10})</td>
<td>1111.9 ± 80.7</td>
<td>Cell adhesion</td>
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<tr>
<td>AA053711</td>
<td>EDIL3</td>
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<td>14.3 ± 0.7</td>
<td>157 (p=9.1e^{-8})</td>
<td>N/D</td>
<td>Cell adhesion</td>
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<tr>
<td>U17496</td>
<td>PSMB8</td>
<td>6</td>
<td>0.91 ± 0.1</td>
<td>0.1 (p=0.01)</td>
<td>N/D</td>
<td>Proteasome subunit</td>
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<td>NM_004666</td>
<td>VNN1</td>
<td>6</td>
<td>0.84 ± 0.1</td>
<td>0.04 (p=0.01)</td>
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<td>Nitrogen metabolism</td>
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<tr>
<td>AU147394</td>
<td>CAV1</td>
<td>7</td>
<td>1.14 ± 0.1</td>
<td>10.9 (p=1.5e^{-4})</td>
<td>15.00 ± 0.8</td>
<td>Integ. plasma membr.</td>
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<tr>
<td>BE552421</td>
<td>MTUS1</td>
<td>8</td>
<td>3.52 ± 0.1</td>
<td>3.4 (p=1.8e^{-6})</td>
<td>N/D</td>
<td>Mitoc. tumor suppressor</td>
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<tr>
<td>S68290</td>
<td>AKR1C1</td>
<td>10</td>
<td>0.94 ± 0.1</td>
<td>11.5 (p=8.2e^{-7})</td>
<td>6.72 ± 0.7</td>
<td>Xenobiotics metabolism</td>
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<tr>
<td>NM_001975</td>
<td>ENO2</td>
<td>12</td>
<td>0.92 ± 0.1</td>
<td>6.0 (p=4.6e^{-6})</td>
<td>3.90 ± 0.1</td>
<td>Glycolysis</td>
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<tr>
<td>AK000345</td>
<td>DHRS2</td>
<td>14</td>
<td>0.97 ± 0.1</td>
<td>0.16 (p=0.01)</td>
<td>N/D</td>
<td>Oxidoreductase</td>
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<tr>
<td>NM_004360</td>
<td>CDH1</td>
<td>16</td>
<td>0.33 ± 0.1</td>
<td>0.19 (p=0.01)</td>
<td>0.15 ± 0.1</td>
<td>Cell adhesion</td>
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<tr>
<td>AL471375</td>
<td>PRKCA</td>
<td>17</td>
<td>1.05 ± 0.1</td>
<td>4.2 (p=1.7e^{-7})</td>
<td>2.55 ± 0.2</td>
<td>Regulation cell cycle</td>
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<td>BQ003811</td>
<td>SLC19A1</td>
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<td>0.84 ± 0.1</td>
<td>0.1 (p=0.01)</td>
<td>N/D</td>
<td>Cell adhesion</td>
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<tr>
<td>NM_001569</td>
<td>IRAK1</td>
<td>X</td>
<td>1.25 ± 0.1</td>
<td>0.26 (p=7.3e^{-3})</td>
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<td>IL1 receptor Kinase</td>
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<tr>
<td>NM_004135</td>
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<td>0.85 ± 0.1</td>
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<td>TCA cycle</td>
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<tr>
<td>NM_001183</td>
<td>ATP6AP1</td>
<td>X</td>
<td>0.68 ± 0.1</td>
<td>0.3 (p=0.01)</td>
<td>N/D</td>
<td>ATP biosynthesis</td>
</tr>
</tbody>
</table>

Selga et al. BMC Medical Genomics 2008, 1:35
Genotyping Analysis-HT29

Chromosome 5
Sensitive Cells

Chromosome 5
Resistant Cells

Resistant Cells Replicate 1

Resistant Cells Replicate 2

Sensitive Cells

serinc5
dhfr
acot12
tmem167a
spz1
msh3
ssbp2
xrcc4
zfye16
rasgrf2
atg10
vcan
fam151b
ckmt2
rps23
hapln1
ankrd34b
zcchc9
loc92270
edil3
Chromosome localization

<table>
<thead>
<tr>
<th>Chromosome 5</th>
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<tbody>
<tr>
<td>80M</td>
</tr>
<tr>
<td>80.5M</td>
</tr>
<tr>
<td>81M</td>
</tr>
<tr>
<td>81.5M</td>
</tr>
<tr>
<td>82M</td>
</tr>
<tr>
<td>82.5M</td>
</tr>
<tr>
<td>83M</td>
</tr>
<tr>
<td>83.5M</td>
</tr>
</tbody>
</table>

- **80M**: ZFYVE16 (16) Zinc Finger, FYVE domain containing 16
- **80.5M**: DHFR (16) Dihydrofolate Reductase
- **80.5M**: MSH3 (5) MutS homolog 3
- **81M**: RASGRF2 (18) Ras protein-specific guanine nucleotide - releasing factor 2
- **81M**: SSBP2 (10) Single-stranded DNA binding protein 2
- **81.5M**: XRCC4 (17) X-ray repair complementing defective repair in Chinese hamster cells 4
- **82M**: HAPLN1 (12) Hyaluronan and proteoglican link protein 1
- **82.5M**: EDIL3 (14) EGF-like repeats and discoidin I-like domains 3

Selga et al. BMC Medical Genomics 2008, 1:35
The combination of siRNA against CAV1 and the overexpression of E-cadherin decreases cell viability.

Selga et al. BMC Medical Genomics 2008, 1:35
Determination of Gene Expression profiles in 6 more cell lines: Analysis of genes in common

- Colon cancer: HT29, CaCo-2
- Breast cancer: MCF7, MDA-MB-468
- Pancreatic cancer: Mia PaCa-2
- Osteosarcoma: Saos-2
- Leukemia: K562
DHFR status in all cell lines studied.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Copy-number</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>16.1 ± 1.4</td>
<td>++</td>
</tr>
<tr>
<td>Caco-2</td>
<td>83.4 ± 8.1</td>
<td>N/D</td>
</tr>
<tr>
<td>MCF-7</td>
<td>58.1 ± 0.8</td>
<td>++++</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>0.9 ± 0.1</td>
<td>N/D</td>
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<tr>
<td>MIA PaCa-2</td>
<td>32.2 ± 2.2</td>
<td>+++</td>
</tr>
<tr>
<td>K562</td>
<td>1.9 ± 0.1</td>
<td>++++</td>
</tr>
<tr>
<td>Saos-2</td>
<td>0.6 ± 0.1</td>
<td>+</td>
</tr>
</tbody>
</table>

No changes in *dhfr* gene copy number

*dhfr* gene amplification

Carlota Oleaga
Hierarchical Clustering of genes and cell lines.

Selga et al. Genome Medicine 2009, 1:83
Venn Diagram comparing colon cancer MTX-resistant cell lines.

HT29

Caco-2

Common genes between cell lines.
Softwares for curated pathways analysis and generation of Biological Association Networks (BANs)

Pathway Assist / Architect
(http://www.genomics.agilent.com)

Ingenuity
(http://www.ingenuity.com/)

Uses Natural Language Processing (NLP) to construct databases of Interactions:

- Regulation
- Promoter Binding
- Metabolism
- Protein Modification
- Binding
- Expression
- Transport
- Member
Pathway analysis with Pathway Architect.

BANs of genes in common among the different cell lines.

Selga et al. Genome Medicine 2009, 1:83
Pathway analysis with Pathway Architect.

BAN of all common genes.

Selga et al. Genome Medicine 2009, 1:83
Work published with BioMed Central in the last 12 months by researchers at Universitat de Barcelona

Order by: Date published / Most viewed (30 days) / Most viewed (past year)

1. Research  Open Access  Highly accessed
Network of differentially expressed genes in human cancer cells resistant to methotrexate
Selga E, Oleaga C, Ramirez S, de Almagro MC, Noé V, Ciudad CJ
Genome Medicine 2009, 1:83 (4 September 2009)
[Abstract] [Full text] [PDF] [PubMed] [Related articles]

2. Research  Open Access  Highly accessed
Effects of cigarette smoke on endothelial function of pulmonary arteries in the guinea pig
Ferrer E, Peinado VI, Díez M, Carrasco JL, Musri MM, Martínez A, Rodríguez-Roisin R, Barberà JA
Respiratory Research 2009, 10:76 (14 August 2009)
[Abstract] [Full Text] [PDF] [PubMed] [Related articles]

3. Original investigation  Open Access
Different modulation by dietary restriction of adipokine expression in white adipose tissue sites in the rat
Romero MM, Fernández-López JA, Esteve M, Alemany M
Cardiovascular Diabetology 2009, 8:42 (30 July 2009)
[Abstract] [Full Text] [PDF] [PubMed] [Related articles]

4. Study protocol  Open Access
Community pharmacist intervention in depressed primary care patients (PRODEFAR study): randomized controlled trial protocol
[Abstract] [Full text] [PDF] [PubMed] [Related articles]
Functional Validation of gene nodes with siRNAs.
## β-catenin-mediated transcriptional activation of the Wnt pathway

### Graphical Representation

The graph demonstrates the transcriptional activation measured by RLU/Protein for different conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>TOPFLASH (µg)</th>
<th>pBATEM2-CDH (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Resistant</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

### Diagram

- **β-CAT**: β-Catenin
- **Cytoplasm**: Location of β-CAT in the cytoplasm
- **Nucleus**: Location of β-CAT in the nucleus
- **Target genes**: DHFR, DKK1, Myc, Cyclin D1, TCF-1, PPARγ, MMP-7...

### Additional Information

- **TOPFLASH**: 3 T-Cell Factor (TCF) binding sites + Luciferase

Selga et al. Genome Medicine 2009, 1:83
UGT1A6

mRNA levels

Copy number

Cristina De Almagro

MCF7

MDA-MB-468

UGT1A6 mRNA levels (% of sensitive cells)

Type 1

Type 2

Sensitive

Resistant

UGT1A4

UGT1A6

DNA Copy number (Relative to sensitive cells)

Sensitive

Resistant

UGT1A4

UGT1A6

DNA Copy number (Relative to sensitive cells)
UGT1A activity

![Graph showing naphthol glucuronidation activity for MDA and MCF-7 cell lines. The graph compares normal and resistant conditions.](image)
UGT induction by MTX

MCF7 S

MDA-MB S
AhR (Aryl hydrocarbon Receptor) binding to UGT1A6 promoter
ARNT (AhR nuclear translocator) binding to UGT1A6 promoter

<table>
<thead>
<tr>
<th></th>
<th>ARNT</th>
<th>ARNT</th>
<th>ARNT</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MCF7-S</td>
<td>MCF7-R</td>
<td>MDA-S</td>
</tr>
<tr>
<td>MTX</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NE</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Gel-Shift

Probe

Supershift

Ab-Arnt - - +
NE - + +

Sensitive
SN-38 (Active Metabolite of Irinotecan) in the presence of MTX

SN-38 (µM)

DMSO 0.01 0.025 0.05 0.01 0.25 0.5 1 5

Control

Cell survival (% of the control)

No MTX

MTX 2·10⁻⁸

* ** *** ****
MicroRNAs: Biogenesis.

Splicing can replace Drosha processing

Modified from Winter Fig.1; Nature Cell Biology rev 2009

Winter Fig.2; Nature Cell Biology rev 2009

Rnase III endonuc.

60-70nt

100-1000nt

microRNA gene or intron

RNA Pol II / III

Transcription

pri-microRNA

Drosha, DGCR8

Cleavage

pre-microRNA

Exportin-5, Ran

Nuclear export

pre-microRNA

Dicer, TRBP

Cleavage

microRNA duplex

Ago2

RISC formation

Mature microRNA

mRNA target cleavage

Translational repression

mRNA deadenylation
MicroRNAs have been related to chemotherapy and drug resistance.

- miR-451 antagonir treatment increases chemosensitivity due to the down-regulation of P-protein (Zhu, 2008)
- miR-21 inhibition increases chemosensitivity to Gemcitabine in cholangiocarcinoma cells and increases the apoptosis induced by topotecan in MCF-7 cells (Blower, 2008)
- Transfection of miR-451 increases chemosensitivity in MCF-7/DOX-resistant cells (Kovalchuk, 2008)
- miR-214 induces cell survival and cis platin resistance in ovarian cancer cells (Yang, 2008)
- miR-34a ectopic expression attenuates resistance to camphothecin in prostate cancer cells (Fujita, 2008)
- ...
Differentially expressed miRNAs

Differentially expressed genes
FC ≥ 2 and p ≤ 0,05 ; Benjamini-Hochberg (1324 genes)

miRNA Target prediction
(TargetScan software) 8394 genes

Putative differentially expressed targets
for differentially expressed miRNAs

201 genes

TargetsScan list
diff expr miRNAs
fc≥2 p≤0,05

All entities

Differentially expressed miRNAs
FC ≥ 2 and p ≤ 0,05 ; Benjamini-Hochberg (226 miRNAs)

Initially selected miRNAs
(10)

Table:

<table>
<thead>
<tr>
<th>miRNA</th>
<th>FCA up</th>
<th>FCA down</th>
<th>Raw Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitive</td>
</tr>
<tr>
<td>hsa-miR-224</td>
<td>7,2</td>
<td>1526,97</td>
<td>93,33</td>
</tr>
<tr>
<td>hsa-miR-1930</td>
<td>4,2</td>
<td>519,18</td>
<td>1110,62</td>
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<tr>
<td>hsa-miR-149</td>
<td>7,1</td>
<td>81,61</td>
<td>280,38</td>
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<tr>
<td>hsa-miR-210</td>
<td>12,2</td>
<td>73,54</td>
<td>415,48</td>
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<tr>
<td>hsa-miR-27b</td>
<td>2,9</td>
<td>492,61</td>
<td>663,36</td>
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<tr>
<td>hsa-miR-320</td>
<td>2,7</td>
<td>309,36</td>
<td>384,22</td>
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<td>hsa-miR-361-5p</td>
<td>2,6</td>
<td>237,71</td>
<td>279,34</td>
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<td>hsa-miR-365</td>
<td>4,5</td>
<td>512,11</td>
<td>1018,93</td>
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<td>hsa-miR-378</td>
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<td>176,50</td>
<td>207,64</td>
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<td>hsa-miR-455-3p</td>
<td>2,7</td>
<td>666,00</td>
<td>811,95</td>
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</tbody>
</table>

Functional validation

Núria Mencia
miRNA-224 and its putative targets

<table>
<thead>
<tr>
<th>miRNA</th>
<th>FCA</th>
<th>Gene</th>
<th>FCA up</th>
<th>FCA down</th>
<th>Raw Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miRNA-224</td>
<td>9,19 down</td>
<td>SLC4A4</td>
<td>2,11</td>
<td>72,58</td>
<td>153,35</td>
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<tr>
<td></td>
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<td>CPNE8</td>
<td>2,52</td>
<td>34,57</td>
<td>9,31</td>
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<td></td>
<td>DIDO1</td>
<td>2,15</td>
<td>228,65</td>
<td>106,29</td>
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<tr>
<td></td>
<td></td>
<td>GPC4</td>
<td>4,33</td>
<td>85,9</td>
<td>19,85</td>
</tr>
</tbody>
</table>

✓ Organic Anion Transporters (OATs, SLC22a6-8) can transport xenobiotics such as β-lactam antibiotics, antiviral drugs, diuretics, NSAIDs, PAH, estrone sulfate, fluorescein and MTX (Methotrexate).

✓ In early stages of MTX resistance, cells present a lower uptake of the drug.
miRNA-224 endogenous expression levels in HT29 cells

Inhibition of miRNA-224 levels by an aODN against miRNA-224 (AntimiR)
Inhibition of miRNA-224...

...decreases sensitivity towards MTX in HT29 sensitive cells.

...increases SLC4A4 expression levels (isoform-specific)

Survival (% of control)

<table>
<thead>
<tr>
<th>Methotrexate (M)</th>
<th>-</th>
<th>3x10^-8</th>
<th>5x10^-8</th>
<th>6x10^-8</th>
<th>8x10^-8</th>
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</thead>
<tbody>
<tr>
<td>Survival (%)</td>
<td>100</td>
<td>90</td>
<td>80</td>
<td>70</td>
<td>60</td>
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</tbody>
</table>

mRNA expression levels (FC)

<table>
<thead>
<tr>
<th>αODN miR-224</th>
<th>-</th>
<th>100nM</th>
<th>1μM</th>
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</thead>
<tbody>
<tr>
<td>miRNA-224</td>
<td>1</td>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>SLC4A4 isoform 1</td>
<td>1</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>SLC4A4 isoform 2</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>SLC4A4 isoform 3</td>
<td>1</td>
<td>1</td>
<td>1</td>
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</tbody>
</table>
DHFR locus Amplification

AKR1C overexpression

Increase in caveolin

UGT1A6 overexpression

Loss of E-Cadherin

Increase in SLC4A4

Resistance To MTX

Increase of Beta-catenin, DKK1, S100A4

Decrease of miR-224
Thanks to the people who did the work: